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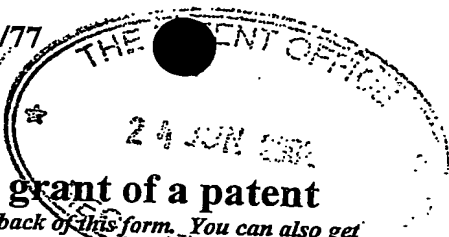
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Dated 11 July 2003



1/77

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POL/7700 0.00-0214528.2

1. Your reference

SMK/BP5956735

2. Patent application number

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0214528.2

24 JUN 2002

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Patents ADP number (if you know it)

The University Court of The University of Aberdeen  
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UNITED KINGDOM

If the applicant is a corporate body, give the country/state of its incorporation

GB

4267126002

4. Title of the invention

Materials and Methods for Induction of Immune Tolerance

5. Name of your agent (if you have one)

MEWBURN ELLIS

Address for service in the United Kingdom to which all correspondence should be sent (including the postcode)

YORK HOUSE  
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Country

Priority application number  
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Date of filing  
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Number of earlier application

Date of filing  
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Claim(s) 8

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11.

I/We request the grant of a patent on the basis of this application.

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*Simon M Kremer*

Date

24 June 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

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## Materials and methods for induction of immune tolerance

### Background to the invention

5 A wide variety of strategies are employed by viruses to evade  
immune mediated clearance, which can be considered to belong to one  
of three major mechanisms: escape, resistance and counterattack (Xu  
et al., 2001). Viruses escape immune recognition by disruption of  
antigen presentation pathways (Lorenzo et al., 2001) and epitope  
10 mutation (Erickson et al., 2001). Resistance is mediated by  
inhibiting apoptosis of virally infected cells. Counterattack  
comprises the killing of effector T cells (Mueller et al., 2001).  
Epstein-Barr virus (EBV) has been shown to avoid detection and  
clearance by such mechanisms. For example, escape of detection in  
Burkitt's lymphoma, where the cells express very low levels of MHC  
15 class I and adhesion molecules (Gregory et al., 1988), and Epstein-  
Barr Nuclear Antigen 1 escapes MHC class I presentation via its NH<sub>2</sub>  
terminal Gly/Ala repeat domain (Levitskaya et al., 1995, 1997).  
EBV also evades immune responses by resistance to apoptosis through  
its Bcl-2 homologue, BHRF-1 (Xu et al., 2001) and expression of the  
20 anti-apoptotic protein A20 induced by LMP1 (Fries et al., 1996).  
However, although EBV has been shown to avoid detection and  
clearance by these, and other mechanisms, it is still not clear how  
the virus maintains latent infection so successfully.

25 EBV is a human  $\gamma$ -herpes virus carried as a latent infection by more  
than 90% of adults, replicating in B-cells and nasopharyngeal  
epithelial cells (Kieff, 1996). The acute infection is controlled  
by a cytotoxic response predominantly against EBV Nuclear Antigens  
3A, 3B and 3C (Kieff, 1996), but, in all cases, the virus enters a  
30 latent state in B-cells (Kieff, 1996). LMP1 is part of a  
restricted panel of genes expressed during latency, and in several  
EBV-associated malignancies including Hodgkin's disease and  
nasopharyngeal carcinoma (Horikawa et al., 2000; Pallesen et al.,  
1991). The protein acts as a constitutively activated tumor  
35 necrosis factor receptor, transforming cells through activation of  
molecules including nuclear factor kappa B and the anti-apoptotic

protein A20 (Eliopoulos et al., 1996, 1997; Huen et al., 1995; Mosialos et al., 1995; Young et al., 1998).

The role of different CD4<sup>+</sup> T helper (Th) cell subsets in regulating the nature and efficacy of immune responses is increasingly recognized (Christensen et al., 2001; Groux et al., 1997; Levings and Roncarolo, 2000; Roncarolo et al., 2000, 2001; Shevach et al., 1998; Stephens and Mason, 2000; Thomsen et al., 2001). Initially, attention focused on mutual antagonism between Th1 and Th2 cells, which produce  $\gamma$ -interferon ( $\gamma$ -IFN) and IL-4 respectively (Mossman and Coffman, 1989), but further, T regulatory (Tr) cell subpopulations with important roles in immunoregulation and tolerance have now been defined (Groux et al., 1997; Levings and Roncarolo, 2000; Roncarolo et al., 2000, 2001; Shevach et al., 1998; Stephens and Mason, 2000). In particular, production of the Tr1 cytokine IL-10 can protect rodents against a number of immune-mediated diseases (Groux et al., 1997; Levings and Roncarolo, 2000), whilst Th3 cell secretion of transforming growth factor- $\beta$  prevents spontaneous autoimmunity (Gorelik and Flavell, 2000) and mediates some forms of oral tolerance (Weiner, 1997). Regulatory subpopulations characterized by CD25 expression have also been isolated from rodents (Seddon, and Mason, 2000; Shevach, 2000), and more recently from human peripheral blood (Jonuleit, et al., 2001; Levings, et al., 2001), but in most reports the suppressive effects of these cells are non-specific and not dependent on cytokine production. The importance of Tr cells in controlling immune-mediated disease raises the prospect that viruses may exploit such regulation as a fourth major mechanism to evade immune clearance.

Given that cells latently infected with EBV express LMP1, the question arises as to why this antigen fails to elicit protective cytotoxic immunity (Chapman et al., 2001; Khanna et al., 1998); cytotoxic T cells specific for LMP1 are notable for their absence from infected individuals (Chapman et al., 2001). Dukers et al. (2000) have recently suggested that LMP1 contains peptide motifs which can direct direct immunosuppressive effects on peripheral

Disclosure of the invention

5 The present inventors have found that certain infectious agents encode antigens comprising tolerogenic peptide sequences. By a "tolerogenic" peptide sequence is meant a sequence which, when administered to cells of the immune system, along with a target antigen, tolerises the cells to that target antigen.

10 Exposure to the tolerogenic sequence and the target antigen inhibits the capacity of the cells to mount an immune response to that target antigen on a subsequent challenge, regardless of whether or not the tolerogenic sequence is present for that subsequent challenge. However, although tolerised to the target  
15 antigen, populations of cells so treated retain their capacity to mount a response to other antigens in the absence of the tolerogenic sequence.

20 The types of immune response which can be inhibited in this way include "defensive" immune responses against foreign antigens, including those administered therapeutically, as well as "pathogenic" immune responses as seen in autoimmune and allergenic diseases. These responses are often characterised by lymphocyte proliferation, expression of cytokines such as IL-4 or gamma-IFN,  
25 and induction of antibody response.

The cells to be tolerised will be from an individual who has previously been infected with the infectious agent from which the tolerogenic peptide is derived.

30 Thus these tolerogenic sequences can induce antigen-specific tolerance of mononuclear leukocytes to target antigens. This activity therefore contrasts with the non-specific immunosuppressive effects attributed to some virus-derived  
35 peptides, e.g. from retroviral envelope proteins (Haraguchi et al., 1995) and EBV LMP1 protein (Dukers et al., 2000).

The present inventors have shown that it is possible to identify such sequences by testing their ability to induce expression of IL-10 in cells from a donor seropositive for the relevant infectious agent.

Accordingly, in a first aspect, the present invention provides a method for assessing the tolerogenicity of a test peptide sequence from an infectious agent, comprising the steps of:

- (i) contacting a cell population with said test peptide sequence,
- (ii) determining whether IL-10 expression in said cell population is increased, and optionally
- (iii) correlating the result of step (ii) with the tolerogenicity of the sequence,

wherein said cell population comprises mononuclear leukocytes from a donor previously infected by said infectious agent.

The term "mononuclear leukocytes" as used herein embraces T lymphocytes (including CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes), B lymphocytes, natural killer (NK) cells, mononuclear phagocytes (monocytes and macrophages) and dendritic cells. Thus the cell population comprises one or more of these types of cells.

Preferably, the cell population comprises at least T lymphocytes, preferably CD4<sup>+</sup> lymphocytes, or at least one type of antigen presenting cell (APC). More preferably, the cell population comprises at least T lymphocytes, preferably CD4<sup>+</sup> lymphocytes, and at least one type of antigen presenting cell. An antigen presenting cell is any cell capable of presenting an antigen to a T lymphocyte in the context of an MHC class II molecule. Thus B lymphocytes, natural killer (NK) cells, mononuclear phagocytes

~~---(monocytes and macrophages) and dendritic cells are all considered~~  
to be APCs. However, the majority of nucleated cells are capable

exposed to pro-inflammatory cytokines, and so the cell population may further comprise APCs which would not normally be regarded as mononuclear leukocytes.

5 The cell population comprises mononuclear leukocytes derived from a donor previously infected by the relevant infectious agent. Preferably it can be demonstrated by an appropriate assay that the donor has previously raised an immune response against the  
10 infectious agent; for example, the donor may be seropositive for the infectious agent, i.e. have circulating antibodies specific for the infectious agent. Under some circumstances the donor may not have circulating antibodies specific for the infectious agent, for example where insufficient time has elapsed since infection for detectable levels of antibodies to be raised, or where a  
15 substantial time has elapsed since infection and antibody levels have fallen below the threshold of detectability. However, the term "seropositive" will be used throughout this specification to refer to any individual previously infected by the relevant infectious agent, regardless of actual serological status, and the  
20 term "seronegative" should be construed accordingly, i.e as referring to an individual not previously infected by the infectious agent.

25 The method may further comprise the steps of:

(i) (a) contacting a similar cell population from a donor not previously infected by said infectious agent with said test peptide sequence, and

30 (ii) (a) determining whether IL-10 expression in said cell population is increased,

and optionally

35 (ii) (b) comparing the results from step (ii) with the results from step (ii) (a).



In step (iii), the individual results, or any combination of the results, from any of steps (ii), (ii)(a) and (ii)(b) may be correlated with the tolerogenicity of the sequence. In general it is considered that the greater the level of IL-10 expression induced in the seropositive population by the test peptide, the more likely it is that the test peptide will be tolerogenic.

Whether or not IL-10 expression is increased may be determined by any appropriate method. Suitable methods include specific detection of IL-10 protein, e.g. by ELISA (Deveraux et al., 2000), flow cytometry (Kreft et al., 1992), non-competitive flow immunoassay (Kjellstrom et al., 2000), immunofluorescence (Scheffold et al., 2000) or immunoblot; by detection of IL-10 mRNA, e.g. by RT-PCR (Blaschke et al., 2000; Demay et al., 1996), or Northern blot; or by bioassay for IL-10 activity (Schlaak et al., 1994).

The present invention further provides a method for assessing the tolerogenicity of a test peptide sequence from an infectious agent towards a target antigen, comprising the steps of:

- (i) contacting a cell population with (a) said test peptide sequence and (b) a target antigen, to make a test composition, and
- (ii) re-contacting the cell population from said test composition with said target antigen.

wherein said cell population comprises mononuclear leukocytes from a donor previously infected by said infectious agent. Preferably, the cell population comprises at least one type of APC, which may or may not be a mononuclear leukocyte, as set out above.

In general the cell population will not be re-contacted with the test peptide in step (ii).

The method may further comprise the steps of:

(iii) assessing the response of said cell population to said target antigen, and optionally

5 (iv) correlating the result of step (iii) with the tolerogenicity of the test peptide sequence.

10 The response of the cell population to the second challenge with the target antigen may be assessed by any method that enables a tolerised population to be distinguished from a non-tolerised population. For example, a response of a non-tolerised population to a foreign antigen would be expected to include one or more of e.g. cell proliferation (typically lymphocyte proliferation), and expression of one or more cytokines (other than IL-10) such as IL-4, IL-2, IL-12 and gamma-IFN. Thus step (iii) may comprise the  
15 assessment of any one of these markers, or of any other suitable marker.

20 The method may be performed *in vivo* or *in vitro*. Preferably the method is performed *in vitro*, e.g. in culture. However the methods may be performed in any suitable model *in vivo*.

25 The purpose of re-contacting the cells with the target antigen in step (ii) is to confirm that the cells have been tolerised to the target antigen by the initial contact of step (i).

30 Therefore it is desirable that for step (ii), the test composition does not still contain appreciable amounts of the test peptide sequence, or of tolerogenic or immunosuppressive factors produced by the cells themselves, which might interfere with any reaction stimulated by the target antigen in step (ii). Therefore, the method may include the step of allowing the cells to rest between steps (i) and (ii), so that the activity of test peptide in the test composition is reduced, the cells are not still expressing tolerogenic factors which would interfere with any reaction in step  
35 (ii), and the activity of residual tolerogenic factors produced by the cells during or in response to the initial tolerogenic

challenge is reduced. IL-10 activity is used herein as a marker for tolerogenic factors generated by the PBMCs in step (i).

When performed *in vitro*, the method may additionally or alternatively comprise the step of washing the cells prior to step (ii). Washing may be performed in conventional fashion. Typically, the cells will be rested after washing. Fresh antigen presenting cells may be added before recontacting the cells with the target antigen in step (ii).

Without wishing to be bound by any particular theory, it is believed that IL-10 may play an effector role in inducing tolerance, so reduction of IL-10 activity may also be achieved by specific neutralisation, e.g. addition of a neutralising factor to the cells, such as a neutralising anti-IL-10 antibody.

The method may further comprise the step of contacting the cell population with a confirmatory antigen unrelated to the test sequence or the target antigen, to confirm that the cells retain their general reactive capability, even though their reactivity to the target antigen has been modified.

Any suitable antigen may be used as the target antigen or confirmatory antigen. These antigens may be primary antigens or recall antigens; that is to say, the cells in the assay may or may not have been exposed to them before. A typical primary antigen for assay use is KLH (keyhole limpet haemocyanin), while for donors previously immunised with Bacille Calmette-Guérin (BCG), purified protein derivative (PPD) from *Mycobacterium tuberculosis* is a suitable recall antigen. T cell mitogens such as Concanavalin A, which are generally regarded as relatively non-specific in their activation of T cells, can also be used as target or confirmatory antigens within the meaning of the present invention. It has been found that PBMCs can be rendered unresponsive to ConA by the techniques described herein, but still retain their ability to respond to other antigens.

A test peptide sequence which is capable of inducing IL-10 expression and/or antigen-specific tolerance in seropositive cells as described above may be regarded as a "tolerogenic peptide sequence".

5 A tolerogenic peptide sequence may therefore be used to modulate an immune response, either *in vivo* or *in vitro*, by administration to suitable seropositive mononuclear leukocytes along with a target antigen. This technique has a number of applications. For  
10 example, it may be used prophylactically, to prevent subsequent development of an inflammatory response to the target antigen, or to inhibit a pre-existing immune reaction to the target antigen.

15 Accordingly, in a further aspect, the present invention provides a method of tolerising a cell population to a target antigen, comprising contacting said cell population with

(a) a tolerogenic peptide sequence from an infectious agent,

20 and

(b) the target antigen,

25 wherein said cell population comprises mononuclear leukocytes from a donor seropositive for said infectious agent.

30 The cell population may be contacted with the tolerogenic peptide sequence and/or the target antigen directly. Alternatively, the cell population may be contacted with the tolerogenic peptide sequence and/or the target antigen indirectly, e.g. via APCs which would not normally be regarded as mononuclear leukocytes, as described above. Thus a population of APCs may be contacted with the tolerogenic peptide sequence and/or the target antigen, and the cell population subsequently contacted with the population of APCs.

35 The tolerogenic peptide and target antigen may be administered to the cell population, or to the population of APCs, either together

or separately, and in any order. Thus it is not intended that the tolerogenic peptide sequence and target antigen must necessarily be administered simultaneously.

5 Any or all of the steps described may be performed *in vitro*, *in vivo*, or *ex vivo*.

Thus all the steps described may be performed *in vitro*, e.g. in culture.

10

In some embodiments, a tolerogenic peptide sequence and a target antigen may be administered directly to a test subject or a subject to be treated, e.g. an individual who has previously been infected by the relevant infectious agent.

15

In alternative embodiments, a tolerogenic peptide sequence and a target antigen may be administered *in vitro* to a cell population comprising mononuclear leukocytes from such an individual. These cells may then be introduced into a test subject, or a subject to be treated, e.g. the subject from whom they were originally derived.

20

25

In alternative embodiments, a tolerogenic peptide sequence and a target antigen may be administered *in vitro* to a population of APCs. The population of APCs may then be contacted *in vitro* with a cell population comprising mononuclear leukocytes from an infected individual. That cell population, or a subset thereof e.g. some or all of the mononuclear leukocytes, may then be introduced into a test subject, or a subject to be treated, e.g. the subject from whom they were originally derived.

0

5

Alternatively, the population of APCs may be administered to a test subject, or a subject to be treated, e.g. the subject from whom they were originally derived. In this case contact between the cell population and the tolerogenic peptide sequence and target antigen takes place *in vivo* via the APCs.

Thus cells or tissues may be removed from a donor individual or individual to be treated, treated with the tolerogenic peptide sequence and a target antigen, and reintroduced to the donor. Suitable cells or tissues include particular type of antigen presenting cells, heterogeneous populations of cells, e.g. peripheral blood lymphocytes or subsets thereof, lymph nodes, etc.

Preferably, the cell population comprises at least T lymphocytes, preferably CD4<sup>+</sup> T lymphocytes. More preferably, the cell population comprises at least T lymphocytes, preferably CD4<sup>+</sup> T lymphocytes, and at least one type of APC. From the above description it can be seen that the cell population to be tolerised, may in some embodiments be considered to comprise cells *in situ* in a test subject or subject to be treated.

The test subject, or subject to be treated will typically be a mammal, and may be a human. In some embodiments, a test subject may be a non-human mammal e.g. a rodent, rabbit, etc. and will typically be seropositive for the infectious agent.

Certain infectious agents do not have animal models that are easy to manipulate. For example, the human pathogen EBV has no animal model. Therefore the test subject may be a non-human mammal with a severe combined immunodeficiency, comprising lymphocytes from a donor of the appropriate species seropositive for the infectious agent. By "severe combined immunodeficiency" is meant a defect in lymphocyte maturation, so that the affected animal has low or undetectable levels of mature T and/or B lymphocytes. The mammal may be a rodent, for example a mouse or rat, such as the SCID mouse. In preferred embodiments the non-human mammal with the severe combined immunodeficiency is reconstituted with human lymphocytes seropositive for EBV, e.g. from a seropositive donor. Suitable techniques are described in Mosier et al. (1988), McCune et al. (1988), Kamel-Reid et al. (1988), and Rowe et al. (1991). Similar techniques may be applied to create animal models of other conditions.

In any of the embodiments of the present invention, the target antigen may be a suitable test antigen as described above, or any antigen to which an inappropriate or undesirable immune response occurs or is likely to occur. Thus the target antigen may be one implicated in a disease state, e.g. a self antigen implicated in an autoimmune condition, such as rheumatoid arthritis, or an allergic state such as hayfever. The target antigen may be a protein, polypeptide or peptide, including an epitope of a protein, or any other suitable entity capable of provoking an immune reaction, such as polysaccharides, lipids, macromolecular complexes, cells, etc.

Examples of auto-immune diseases in which specific antigens have been identified as potentially pathogenically significant include multiple sclerosis (myelin basic protein), insulin-dependent diabetes mellitus (glutamic acid decarboxylase), insulin-resistant diabetes mellitus (insulin receptor), coeliac disease (gliadin), bullous pemphigoid (collagen type XVII), auto-immune haemolytic anaemia (Rh protein), auto-immune thrombocytopenia (GpIIb/IIIa), myasthenia gravis (acetylcholine receptor), Graves' disease (thyroid-stimulating hormone receptor), glomerulonephritis (collagen type IV), pernicious anaemia (intrinsic factor). Thus these antigens, or particular fragments or epitopes thereof may be suitable target antigens.

The target antigen may be an exogenous antigen which stimulates a response which also causes damage to host tissues. For example, acute rheumatic fever is caused by an antibody response to a Streptococcal antigen which cross-reacts with a cardiac muscle cell antigen. The target antigen may be one which provokes an atopic or allergic response, e.g. pollen (implicated in hayfever, e.g. Timothy Grass pollen), house dust mites (asthma), cosmetics, allergens administered via insect bites, nut allergens, or therapeutic products such as factor VIII, factor IX, blood group antigens, or monoclonal antibodies.

The methods of the present invention may be used to suppress

primary and secondary mixed lymphocyte reactions, graft rejection, and graft versus host disease. Thus a subject intended to receive a cellular transplant may be tolerised to antigens expressed by those cells. Alternatively, the transplant may be given in conjunction with tolerogenic peptide sequences as described herein, or nucleic acid encoding such peptide sequences, in order to tolerise the recipient to those cells. In preferred embodiments, some or all of the cells to be transplanted may be engineered to express tolerogenic peptides. Thus a cell to be transplanted may contain nucleic acid encoding a tolerogenic peptide sequence according to the present invention such that the cell is capable of expressing the tolerogenic peptide sequence. The optimum methodology will depend on the identity of the cells to be engineered. Antigen presenting cells, e.g. dendritic cells, etc., may be engineered to express the tolerogenic peptide sequence in such a manner that it is processed and presented in the context of the cells' own MHC II molecules. Other cell types may be engineered so that they secrete the expressed sequence, in order that it can be presented by neighbouring APCs.

In all of the aspects described herein, the infectious agent, from which the test or tolerogenic peptide sequence is derived, may be a virus. In preferred embodiments, the virus is a herpesvirus encoding a viral IL-10 homologue, preferably EBV.

The test or tolerogenic peptide sequence may be derived from an EBV protein, preferably EBV LMP1 protein or LMP2 protein. Thus the methods of the present invention extend to the use of LMP1 protein, LMP2 protein, or a portion or fragment thereof comprising a tolerogenic peptide sequence.

The test or tolerogenic peptide sequence may comprise one or more of the sequences p1 to p75, or p1' to p96'. If desired, more than one test or tolerogenic peptide sequence may be administered, either simultaneously or sequentially.



The present invention also provides a method of treating a disease mediated by an immune response against a target antigen, comprising the steps of administering (a) a tolerogenic peptide sequence from an infectious agent, and (b) the target antigen, to an individual seropositive for said infectious agent.

Nucleic acids encoding test or tolerogenic peptides, and/or target antigens, may be useful in all the methods of the present invention. As an alternative to administration of a peptide to cells, a nucleic acid encoding that peptide and capable of supporting its expression may be used instead. For example, DNA vaccination techniques are well known to the skilled person, as reviewed in Mor and Eliza (2001); Smith (2000); Schleef et al. (2000) and Apostolopoulos and Plebanski (2000). Thus where administration of a peptide sequence is referred to in any of the methods herein described, administration of a nucleic acid sequence encoding that peptide sequence is also envisaged. Thus contacting a cell population or population of antigen presenting cells with a peptide sequence is considered to encompass contacting the relevant cells with an appropriate nucleic acid.

Thus for example, the present invention further provides a method of tolerising a cell population to a target antigen, comprising contacting said cell population with

(a) a nucleic acid encoding said test peptide sequence, such that said test peptide sequence is expressed in said cell population, and

(b) the target antigen,

wherein said cell population comprises mononuclear leukocytes from a donor seropositive for said infectious agent.

Where the target antigen is a protein, polypeptide or peptide, a nucleic acid encoding the target antigen may be administered to the individual.

However this should not be taken to imply that the target antigen need necessarily be a protein, polypeptide or peptide.

5 Use of nucleic acids in this way is considered to be applicable, *mutatis mutandis*, to any corresponding embodiment of the present invention in which administration of a peptide sequence is referred to. When target antigens are protein or peptide, nucleic acids having appropriate coding sequences may likewise be administered instead. In related embodiments, cells may be contacted with  
10 peptides by contact with cells engineered to express the relevant peptides and either secrete them or present them in the context of MHC molecules.

15 The present invention further provides a pharmaceutical composition comprising a tolerogenic peptide sequence from an infectious agent and a target antigen, in admixture with a pharmaceutically acceptable carrier.

20 In preferred embodiments, the tolerogenic peptide sequence is derived from EBV, e.g. LMP1 or LMP2 as described above. Thus the composition may comprise EBV LMP1 protein, LMP2 protein, or a portion or fragment of either comprising a tolerogenic peptide sequence.

25 In preferred embodiments the tolerogenic peptide sequence may comprise one or more of the LMP1 peptide sequences P1 to P75, and/or one or more of the LMP2 peptide sequences P1' to P96' described herein.

30 The present invention further provides EBV LMP1 and LMP2 proteins, and portions or fragments of either, for example, the peptide sequences P1 to P75, or P1' to P96' comprising a tolerogenic peptide sequence, for use in a method of medical treatment.

35 The present invention further provides EBV LMP1 and LMP2 proteins, and portions or fragments thereof, for example, the peptide sequences P1 to P75, or P1' to P96' comprising a tolerogenic

peptide sequence, for use in the treatment of a condition mediated by an immune response directed against a target antigen.

The present invention further provides EBV LMP1 and LMP2 proteins, and portions or fragments thereof, for example, the LMP1 peptide sequences P1 to P75, and the LMP2 peptide sequences P1' to P96' comprising a tolerogenic peptide sequence, in the preparation of a medicament for the treatment of a condition mediated by an immune response directed against a target antigen. The medicament may further comprise the target antigen. The medicament will typically be formulated for administration to an individual previously infected by EBV.

In these and other aspects of the present invention, preferred peptides include P2, P4, P5, P6, P7, P8, P9, P10, P12, P13, P14, P15, P16, P17, P18, P20, P22, P23, P24, P25, P26, P27, P29, P30, P32, P34, P35, P39, P68, P71, P72. Particularly preferred peptides include P2, P4, P7, P14, P15, P18, P20, P22, P23, P24, and P32.

The condition may be, for example, type I diabetes mellitus, coeliac disease, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, myaesthesia gravis, autoimmune haemolytic anaemia and thrombocytopenia, an atopic response e.g. hay fever or asthma, or other allergy, e.g. to an allergen such as a pharmaceutical product or nut allergens, or an alloimmune response, e.g. graft rejection, graft versus host disease, or a response to therapeutic products such as factor VIII, or monoclonal antibody therapy. The target antigens described above may be useful for treatment of these conditions.

The compositions and medicaments described herein may comprise nucleic acids encoding tolerogenic peptides and/or target antigens, as appropriate.

Also provided is a pharmaceutical composition comprising a cell for treatment of a condition in a recipient in combination with a

acid encoding a tolerogenic peptide according to the present invention, such that said tolerogenic peptide sequence can be expressed by said cell.

- 5 The nucleic acid preferably encodes an EBV protein, e.g. LMP1 or LMP2, or a fragment thereof comprising a tolerogenic peptide sequence.

10 The tolerogenic peptide sequence and the target antigen may be administered together or separately. In preferred embodiments, they are administered together. They may be provided as an admixture of separate components, as a complex, or covalently associated. Where the target antigen is a protein, the tolerogenic peptide sequence and target antigen may be provided as a fusion  
15 protein. Use of fusion proteins in this manner is applicable to all aspects of the invention.

In any of the above-described aspects of the invention, the cell population to be tolerised may comprise mononuclear leukocytes from  
20 any suitable species. In preferred embodiments the mononuclear leukocytes are mammalian, e.g. from livestock animals such as horses, cattle, etc., from domestic animals, such as dogs, cats, etc., or from humans. Likewise, individuals to be treated by the methods of the present invention are preferably mammals, e.g.  
25 livestock animals such as horses, cattle, etc., domestic animals, such as dogs, cats, etc., and humans.

The term "peptide sequence" as used herein, whether a test or tolerogenic peptide sequence, should not be taken to refer solely  
30 to a free peptide consisting essentially or exclusively of that sequence, although this is encompassed by the present invention. Without wishing to be bound by any particular theory, it is believed that the methods of the present invention are effective as long as the relevant sequence can be presented to T cells by  
35 antigen presenting cells within the population. Thus it is believed that the test or tolerogenic peptide sequence may constitute a T cell epitope, in that it is capable of being

presented to T cells in the context of MHC molecules. Therefore the test or tolerogenic peptide sequence is preferably at least 6 amino acids in length, more preferably at least 8 amino acids in length.

Preferably the test or tolerogenic peptide sequence is capable of acting as an MHC class II-restricted T cell epitope. The chance that a peptide will be capable of acting as a T cell epitope can be determined by assessing its ability to bind to the antigen binding groove of MHC II molecules. Peptide motifs which bind particular MHC alleles are known, and computer programs are available which can identify such motifs within protein sequences (Sturniolo et al. (1999); Singh and Raghava (2001)).

The skilled person will be aware that any T cell that responds to a given peptide can also respond in a similar way to other peptides containing substitutions in residues that are not critical for MHC binding or T cell receptor recognition, and even to certain peptides that are substituted in critical residues. Such immunological cross reactivity of peptides can be demonstrated by showing that a particular T cell is capable of responding to more than one peptide. Such experiments may be performed using T cell clones. Techniques for cloning T cells are well known in the art. Without wishing to be bound by any particular theory, T cells of Tr1 phenotype may be implicated in the mechanism underlying the methods described herein. Such T cells do not proliferate significantly in response to stimulation, and suppress proliferation of other cells, and so can be difficult to clone. However, suitable techniques are known - see e.g. MacDonald et al. (2002).

Tolerogenic peptides derived from infectious agents described herein, or identified using the methods herein, may be used to screen for immunologically cross reactive peptides which exert similar tolerogenic effects by stimulating a similar or overlapping T cell population. Such cross reactive peptides may be considered

described herein. Thus the present invention provides a method for assessing the tolerogenicity of a test peptide sequence, comprising the steps of:

5 (i) contacting a first cell population with said test peptide sequence,

(ii) contacting a second cell population with a control peptide sequence

10 (iii) determining whether IL-10 expression in each said cell population is increased, and optionally

15 (iv) correlating the result of step (iii) with the tolerogenicity of the test peptide sequence,

wherein each said cell population comprises mononuclear leukocytes from a donor previously infected by an infectious agent, and said control peptide sequence is derived from said infectious agent.

20 Thus typically, the control peptide sequence will have been previously shown to induce IL-10 expression in a cell population comprising mononuclear leukocytes from a donor previously infected by said infectious agent.

25 Preferably, the first and second cell populations are derived from the same donor individual. In preferred embodiments the first and second cell populations comprise T cell clones, preferably Tr1 T cell clones, shown to respond to the control peptide when appropriately presented by APCs.

30 The control peptide may comprise one or more of peptides P1 to P75 and/or P1' to P96' described herein.

35 The skilled person will also be aware that, because of the polymorphic nature of the MHC, most peptides will not be capable of binding to all MHC molecules. Thus compositions for use in the present invention may be tailored to a specific individual, by

selecting peptides likely to bind to their MHC. Alternatively, compositions may be designed to have a broader spectrum of activity, being applicable to a wider range of the population. This may be achieved by incorporating peptides capable of binding more than one MHC allele, and/or incorporating more than one test or tolerogenic peptide, each having different MHC specificity. These peptides may be provided in any appropriate form, e.g. as mixtures of separate peptides or as fusion proteins.

Therefore the test or tolerogenic peptide sequence may be administered as part of a longer peptide, polypeptide or protein. For example, the sequence may be used in the context of the whole or part of the full length native protein. The peptide, polypeptide or protein may be administered in any appropriate form, e.g. in native or denatured conformation.

It will be appreciated that any peptide, polypeptide or protein may comprise more than one tolerogenic peptide sequence within the meaning of the present invention. For example, the EBV LMP1 protein is believed to contain numerous individual peptide sequences capable of inducing tolerance to a target antigen in EBV-seropositive PBMCs, as described more fully in the Examples below.

Furthermore, a peptide, polypeptide or protein comprising one or more tolerogenic epitopes may be utilised in admixture with target antigen, or may, for example, be provided covalently coupled with a target antigen, either by chemical linkage, or, where the target antigen is a protein, as a fusion protein.

Peptides, polypeptides or proteins, including fusion proteins, for use in the methods or compositions of the present invention may be generated by any appropriate method, including chemical synthesis and recombinant expression.

The present invention further provides individual peptides having any one of the sequences of SEQ ID NO: 1 to 100, 101 to 200, 201 to 300, 301 to 400, 401 to 500, 501 to 600, 601 to 700, 701 to 800, 801 to 900, 901 to 1000, 1001 to 1100, 1101 to 1200, 1201 to 1300, 1301 to 1400, 1401 to 1500, 1501 to 1600, 1601 to 1700, 1701 to 1800, 1801 to 1900, 1901 to 2000, 2001 to 2100, 2101 to 2200, 2201 to 2300, 2301 to 2400, 2401 to 2500, 2501 to 2600, 2601 to 2700, 2701 to 2800, 2801 to 2900, 2901 to 3000, 3001 to 3100, 3101 to 3200, 3201 to 3300, 3301 to 3400, 3401 to 3500, 3501 to 3600, 3601 to 3700, 3701 to 3800, 3801 to 3900, 3901 to 4000, 4001 to 4100, 4101 to 4200, 4201 to 4300, 4301 to 4400, 4401 to 4500, 4501 to 4600, 4601 to 4700, 4701 to 4800, 4801 to 4900, 4901 to 5000, 5001 to 5100, 5101 to 5200, 5201 to 5300, 5301 to 5400, 5401 to 5500, 5501 to 5600, 5601 to 5700, 5701 to 5800, 5801 to 5900, 5901 to 6000, 6001 to 6100, 6101 to 6200, 6201 to 6300, 6301 to 6400, 6401 to 6500, 6501 to 6600, 6601 to 6700, 6701 to 6800, 6801 to 6900, 6901 to 7000, 7001 to 7100, 7101 to 7200, 7201 to 7300, 7301 to 7400, 7401 to 7500, 7501 to 7600, 7601 to 7700, 7701 to 7800, 7801 to 7900, 7901 to 8000, 8001 to 8100, 8101 to 8200, 8201 to 8300, 8301 to 8400, 8401 to 8500, 8501 to 8600, 8601 to 8700, 8701 to 8800, 8801 to 8900, 8901 to 9000, 9001 to 9100, 9101 to 9200, 9201 to 9300, 9301 to 9400, 9401 to 9500, 9501 to 9600, 9601 to 9700, 9701 to 9800, 9801 to 9900, 9901 to 10000, 10001 to 10100, 10101 to 10200, 10201 to 10300, 10301 to 10400, 10401 to 10500, 10501 to 10600, 10601 to 10700, 10701 to 10800, 10801 to 10900, 10901 to 11000, 11001 to 11100, 11101 to 11200, 11201 to 11300, 11301 to 11400, 11401 to 11500, 11501 to 11600, 11601 to 11700, 11701 to 11800, 11801 to 11900, 11901 to 12000, 12001 to 12100, 12101 to 12200, 12201 to 12300, 12301 to 12400, 12401 to 12500, 12501 to 12600, 12601 to 12700, 12701 to 12800, 12801 to 12900, 12901 to 13000, 13001 to 13100, 13101 to 13200, 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P7, P8, P9, P10, P12, P13, P14, P15, P16, P17, P18, P20, P22, P23, P24, P25, P26, P27, P29, P30, P32, P34, P35, P39, P68, P71, P72. Particularly preferred peptides have sequences of P2, P4, P7, P14, P15, P18, P20, P22, P23, P24, and P32.

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Thus in a further aspect, the present invention provides isolated nucleic acid molecules encoding the test and tolerogenic sequences of the present invention. The open reading frame may be contiguous with an open reading frame encoding a desired target antigen, in order to encode a fusion protein as described above.

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In further aspects, the present invention provides an expression vector comprising the above tolerogenic sequence-encoding nucleic acid, operably linked to control sequences to direct its expression, as well as host cells transformed with the vectors. The present invention also includes a method of producing peptides of the preceding aspect, comprising culturing the host cells and isolating the tolerogenic peptides thus produced.

15

In order to obtain expression of nucleic acids encoding test, tolerogenic or target antigen sequences, the sequences can be incorporated into a vector having control sequences operably linked to the encoding nucleic acid to control its expression. The vectors may include other sequences such as promoters or enhancers to drive the expression of the inserted nucleic acid, nucleic acid sequences so that the tolerogenic sequence peptide is produced as a fusion, e.g. with one or more other such tolerogenic sequences, or with one or more target antigens, and/or nucleic acid encoding secretion signals so that the peptide produced in the host cell is secreted from the cell. Peptides/polypeptides/proteins can then be obtained by transforming the vectors into host cells in which the vector is functional, culturing the host cells so that the peptide is produced and recovering the peptide from the host cells or the surrounding medium. Prokaryotic and eukaryotic cells are used for this purpose in the art, including strains of *E. coli*, yeast, and eukaryotic cells such as COS or CHO cells.

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Suitable vectors can be chosen or constructed, containing appropriate regulatory sequences, including promoter sequences, terminator fragments, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate.

Vectors may be plasmids, viral e.g. 'phage, or phagemid, as appropriate. For further details see, for example, "Molecular Cloning: a Laboratory Manual": 2nd edition, Sambrook et al., 1989, Cold Spring Harbor Laboratory Press.

Cells and techniques may be selected such as to permit or enhance the folding and/or formation of disulphide bridges (see e.g. "Protein Folding" by R. Hermann, Pub. 1993, European Patent Office, The Hague, Netherlands, ISBN 90-9006173-8).

Peptides may be synthesized by any suitable method, such as by exclusively solid-phase techniques, by partial solid-phase techniques, by fragment condensation or by classical solution couplings. In conventional solution phase peptide synthesis, the peptide chain can be prepared by a series of coupling reactions in which the constituent amino acids are added to the growing peptide chain in the desired sequence.

Briefly, N-alpha-protected amino acid anhydrides are prepared in crystallized form or prepared freshly in solution and used for successive amino acid addition at the N-terminus. At each residue addition, the growing peptide (on a solid support) is acid treated to remove the N-alpha-protective group, washed several times to remove residual acid and to promote accessibility of the peptide terminus to the reaction medium. The peptide is then reacted with an activated N-protected amino acid symmetrical anhydride, and the solid support is washed. At each residue-addition step, the amino acid addition reaction may be repeated for a total of two or three separate addition reactions, to increase the percent of growing peptide molecules which are reacted. Typically, 1-2 reaction cycles are used for the first twelve residue additions, and 2-3 reaction cycles for the remaining residues.

The use of various N-protecting groups, various coupling reagents, e.g., dicyclohexylcarbodiimide or carbonyldiimidazole, various active esters, e.g., esters of N-hydroxyphthalimide or N-hydroxysuccinimide, and the various cleavage reagents, to carry out reaction in solution, with subsequent isolation and purification of intermediates, is well known classical peptide methodology. Classical solution synthesis is described in detail in the treatise "Methoden der Organischen Chemie (Houben-Weyl): Synthese von Peptiden", E. Wunsch (editor) (1974) Georg Thieme Verlag, Stuttgart, W. Ger. Techniques of exclusively solid-phase synthesis are set forth in the textbook "Solid-Phase Peptide Synthesis", Stewart & Young, Pierce Chemical Co., Rockford, Ill., 1984, and are exemplified by the disclosure of U.S. Pat. No. 4,105,603. The fragment condensation method of synthesis is exemplified in U.S. Pat. No. 3,972,859. Other available syntheses are exemplified by U.S. Pat. Nos. 3,842,067 and 3,862,925.

Peptides are preferably prepared using the Merrifield solid phase synthesis, although other equivalent chemical syntheses known in the art can also be used as previously mentioned. Such solid-phase synthesis is commenced from the C-terminus of the peptide by coupling a protected *alpha*-amino acid to a suitable resin. Such a starting material can be prepared by attaching an *alpha*-amino-protected amino acid by an ester linkage to a chloromethylated resin or a hydroxymethyl resin, or by an amide bond to a benzhydrylamine (BHA) resin or paramethylbenzhydrylamine (MBHA) resin. The preparation of the hydroxymethyl resin is described by Bodansky et al., Chem. Ind. (London) 38, 1597-98 (1966). Chloromethylated resins are commercially available from Bio Rad Laboratories, Richmond, Calif. and from Lab. Systems, Inc. The preparation of such a resin is described by Stewart et al., "Solid Phase Peptide Synthesis", supra.

The C-terminal amino acid, protected by Boc and by a side-chain protecting group, if appropriate, can be first coupled to a chloromethylated resin according to the procedure set forth in Chemistry Letters, K. Horiki et al. 165-168 (1978), using KF in DMF

at about 60°C. for 24 hours with stirring, when a peptide having free acid at the C-terminus is to be synthesized.

5 Conditions for removal of specific *alpha*-amino protecting groups may be used as described in Schroder & Lubke, "The Peptides", 1 pp 72-75, Academic Press (1965).

10 Activating reagents and their use in peptide coupling are described by Schroder & Lubke supra, in Chapter III and by Kapoor, J. Phar. Sci., 59, pp 1-27 (1970).

15 The success of the coupling reaction at each stage of the synthesis, if performed manually, is preferably monitored by the ninhydrin reaction, as described by E. Kaiser et al., Anal. Biochem. 34, 595 (1970). The coupling reactions can be performed automatically, as on a Beckman 990 automatic synthesizer, using a program such as that reported in Rivier et al. Biopolymers, 1978, 17, pp 1927-1938.

20 After completing the growing peptide chains, the protected peptide resin is treated with liquid hydrofluoric acid to deblock and release the peptides from the support. For preparing an amidated peptide, the resin support used in the synthesis is selected to supply a C-terminal amide, after peptide cleavage from the resin.  
 25 After removal of the hydrogen fluoride, the peptide is extracted into 1M acetic acid solution and lyophilized.

0 The peptide can be isolated by an initial separation by gel filtration, to remove peptide dimers and higher molecular weight polymers, and also to remove undesired salts.

5 Test and tolerogenic peptide sequences need not correspond exactly to the amino acid sequence of the agent infecting the host from which the PBMCs to be tolerated are derived. It is well known that proteins from wild type isolates of infectious agents often contain differences relative to the sequence of reference peptides of that

agent. However, use of peptides synthesised according to reference sequences will typically provide the desired tolerogenic effects.

5 In some circumstances, it may be desirable and feasible to use a test or tolerogenic sequence not from the agent infecting the host, but from a related agent, as long as the agents are sufficiently closely related for immunological cross-reactivity to occur, such that the desired tolerance is induced.

10 Alternatively, it may be desirable deliberately to introduce sequence mutations relative to either a wild type isolate or reference isolate. For example, without wishing to be bound by any particular theory, it is believed that the test/tolerogenic sequences may exert their effects by being presented to T cells  
15 with a Tr1 phenotype (3) by antigen presenting cells. Therefore it may be desirable to introduce mutations into a tolerogenic peptide from a given infectious agent in order to enable it to bind to a broader range of MHC molecules, and thus be used to tolerise a larger proportion of a population towards target antigens.

20 Therefore test or tolerogenic peptides may be used which differ from known or wild type sequences for the corresponding region of the infectious agent protein, as long as they retain sufficient tolerogenic capability. This can readily be determined by use of  
25 the methods of the present invention.

Variant peptides can be produced by a mixture of conservative variation, i.e. substitution of one hydrophobic residue such as isoleucine, valine, leucine or methionine for another, or the  
30 substitution of one polar residue for another, such as arginine for lysine, glutamic for aspartic acid, or glutamine for asparagine. As is well known to those skilled in the art, altering the primary structure of a polypeptide by a conservative substitution may not significantly alter the activity of that peptide because the  
35 side-chain of the amino acid which is inserted into the sequence may be able to form similar bonds and contacts as the side chain of the amino acid which has been substituted out. This is so even when

the substitution is in a region which is critical in determining peptide conformation. Also included are variants having non-conservative substitutions. As is well known to those skilled in the art, substitutions to regions of a peptide which are not critical in determining its conformation may not greatly affect its activity because they do not greatly alter the peptide's three dimensional structure, and so may not affect the desired activity, e.g. MHC binding. In regions which are critical in determining the peptides conformation or activity such changes may confer advantageous properties on the polypeptide. Indeed, changes such as those described above may confer slightly advantageous properties on the peptide e.g. altered stability or specificity.

Generally variant peptides may be extended at the N- or C-termini, and the C-terminus may be amidated or have a free acid form.

A peptide which is an amino acid sequence variant will generally share at least about 50%, 60%, 70%, 80%, 90% or more sequence identity with a wild type or reference sequence from the relevant infectious agent. In this connection, "sequence identity" means strict amino acid identity between the sequences being compared.

Once an amino acid substitution or other modification is made as described above, the peptide is screened for the requisite tolerogenic activity, as described above.

As described above, compositions of the present invention may comprise, in addition to the tolerogenic peptide sequences and optionally target antigens, a pharmaceutically acceptable excipient, carrier, buffer, stabiliser or other materials well known to those skilled in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The precise nature of the carrier or other material may depend on the route of administration, e.g. oral, intravenous, cutaneous or subcutaneous, nasal, intramuscular, intraperitoneal

Pharmaceutical compositions for oral administration may be in tablet, capsule, powder or liquid form. A tablet may include a solid carrier such as gelatin or an adjuvant. Liquid pharmaceutical compositions generally include a liquid carrier such as water, petroleum, animal or vegetable oils, mineral oil or synthetic oil. Physiological saline solution, dextrose or other saccharide solution or glycols such as ethylene glycol, propylene glycol or polyethylene glycol may be included as required.

As the compositions of the present invention comprise peptides as active agents, they will typically be delivered by other routes, e.g. by intravenous, cutaneous or subcutaneous injection, or injection at the site of affliction, when the active ingredient will be in the form of a parenterally acceptable aqueous solution which is pyrogen-free and has suitable pH, isotonicity and stability. Those of relevant skill in the art are well able to prepare suitable solutions using, for example, isotonic vehicles such as Sodium Chloride Injection, Ringer's Injection, Lactated Ringer's Injection. Preservatives, stabilisers, buffers, antioxidants and/or other additives may be included, as required.

For delayed release, the active agents, e.g. tolerogenic peptide sequences and target antigens, may be included in a pharmaceutical composition formulated for slow release, such as in microcapsules formed from biocompatible polymers or in liposomal carrier systems according to methods known in the art.

For continuous release of peptides, the peptides may be covalently conjugated to a water soluble polymer, such as a polylactide or biodegradable hydrogel derived from an amphipathic block copolymer, as described in U.S. Pat. No. 5,320,840. Collagen-based matrix implants, such as described in U.S. Pat. No. 5,024,841, are also useful for sustained delivery of peptide therapeutics. Also useful, particularly for subdermal slow-release delivery, is a composition that includes a biodegradable polymer that is self-curing and that forms an implant in situ, after delivery in liquid form. Such a

composition is described, for example in U.S. Pat. No. 5,278,202.

Thus in a further aspect, the present invention provides a pharmaceutical composition comprising a tolerogenic peptide-  
5 encoding nucleic acid molecule and its use in methods of therapy or diagnosis. The composition may further comprise a target antigen-encoding nucleic acid molecule, which may be contiguous with the tolerogenic peptide-encoding nucleic acid molecule.

10 In a further aspect, the present invention provides a pharmaceutical composition comprising one or more tolerogenic peptide sequences as defined above and its use in methods of therapy or diagnosis. The composition may further comprise one or  
15 more target antigens.

In further aspects, the present invention provides the above described tolerogenic peptide sequences and encoding nucleic acid molecules for use in the preparation of medicaments for therapy.

20 Peptides may preferably be administered by transdermal iontophoresis. One particularly useful means for delivering compounds is transdermal delivery. This form of delivery can be effected according to methods known in the art. Generally,  
transdermal delivery involves the use of a transdermal "patch"  
5 which allows for slow delivery of compound to a selected skin region. Such patches are generally used to provide systemic delivery of compound. Examples of transdermal patch delivery systems are provided by U.S. Pat. No. 4,655,766 (fluid-imbibing osmotically driven system), and U.S. Pat. No. 5,004,610 (rate  
0 controlled transdermal delivery system).

For transdermal delivery of peptides, transdermal delivery may preferably be carried out using iontophoretic methods, such as described in U.S. Pat. No. 5,032,109 (electrolytic transdermal  
5 delivery system), and in U.S. Pat. No. 5,314,502 (electrically  
transdermal delivery system).

For transdermal delivery, it may be desirable to include permeation enhancing substances, such as fat soluble substances (e.g., aliphatic carboxylic acids, aliphatic alcohols), or water soluble substances (e.g., alkane polyols such as ethylene glycol, 1,3-propanediol, glycerol, propylene glycol, and the like). In addition, as described in U.S. Pat. No. 5,362,497, a "super water-absorbent resin" may be added to transdermal formulations to further enhance transdermal delivery. Examples of such resins include, but are not limited to, polyacrylates, saponified vinyl acetate-acrylic acid ester copolymers, cross-linked polyvinyl alcohol-maleic anhydride copolymers, saponified polyacrylonitrile graft polymers, starch acrylic acid graft polymers, and the like. Such formulations may be provided as occluded dressings to the region of interest, or may be provided in one or more of the transdermal patch configurations described above.

In other treatment methods, the modulators may be given orally or by nasal insufflation, according to methods known in the art. For administration of peptides, it may be desirable to incorporate such peptides into microcapsules suitable for oral or nasal delivery, according to methods known in the art.

Administration is preferably in a "prophylactically effective amount" or a "therapeutically effective amount" (as the case may be, although prophylaxis may be considered therapy), this being sufficient to show benefit to the individual. The actual amount administered, and rate and time-course of administration, will depend on the nature and severity of what is being treated. Prescription of treatment, e.g. decisions on dosage etc, is within the responsibility of general practitioners and other medical doctors, and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of the techniques and protocols mentioned above can be found in Remington's Pharmaceutical Sciences, 16th edition, Osol, A. (ed), 1980.



Alternatively, targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibody or cell specific ligands. Targeting may be desirable for a variety of reasons; for example if the agent is unacceptably toxic, or if it would otherwise require too high a dosage, or if it would not otherwise be able to enter the target cells.

Instead of administering these agents directly, they could be produced in the target cells by expression from an encoding gene introduced into the cells, e.g. in a viral vector. The vector could be targeted to the specific cells to be treated, or it could contain regulatory elements which are switched on more or less selectively by the target cells.

A composition may be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated.

Specific embodiments of the invention will now be described in more detail, by way of example and not limitation, by reference to the accompanying drawings.

#### Brief description of the drawings

Figure 1 shows cytokine and proliferative responses of PBMC from healthy EBV seropositive and seronegative donors (19) to purified LMP1 (20). Representative results are shown from two EBV seropositive donors (n=10) and two seronegative donors.

Figure 2 shows cytokine and proliferative responses of PBMC from EBV seropositive donors to a panel of LMP1 peptides. Representative results obtained from one donor (n=20) are shown for cytokine ELISAs (IL-10, IL-4, gamma-IFN) and a proliferation assay. The broken line on each chart shows the minimum level considered to be a positive response (0.01).

Figure 3 shows a summary of the percentage of EBV seropositive donors (n=20) whose PBMC responded to each LMP1 peptide with cytokine secretion (IL-10, IL-4, gamma-IFN) or proliferation. The results were demonstrated to be reproducible by retesting all of the 18 available donors.

Figure 4 shows flow cytometric analysis (23) of the phenotype of IL-10 synthesizing cells. After gating on CD3<sup>+</sup> cells, cultured cells from two EBV seropositive donors (A+B and C+D) were analyzed for expression of CD4 and IL-10, with the % of double positive cells shown in the upper right quadrant of each panel. A+C were obtained from unstimulated cultures and B+D from cells stimulated with peptides P14 (aa 66-85) and P8 (aa 36-55) respectively (shown to induce IL-10 in these donors).

Figure 5 shows proliferative and  $\gamma$ -IFN responses by PBMC from EBV seropositive, but not seronegative, donors against a mitogen (Con A), a recall antigen (PPD) and a primary antigen (KLH) (33) in the presence and absence of LMP1. Representative results are shown from two EBV seropositive donors (n=10) and two seronegative donors.

Figure 6 shows that IL-10 inducing LMP1 peptides inhibit proliferative responses by PBMC from EBV seropositive donors against recall antigen (PPD). The white bars show the proliferative and gamma-IFN responses obtained when PBMC from three EBV seropositive donors were stimulated with PPD, either alone, or together with IL-10 inducing LMP1 peptides (P4,7,23,35 for Donor 1, P4 and 22 for Donor 2, and P4,18 and 31 for Donor 3), or control gamma-IFN inducing LMP1 peptides (P28 for Donor 1, P56 for Donor 2 and P33 for Donor 3). The black bars show the effects of adding a neutralizing anti-IL-10 antibody to duplicate cultures at 0.5 $\mu$ g/ml.

Figures 7 to 10 illustrate the specificity and persistence of LMP-1-induced tolerance. In each Figure, panel (a) shows proliferative,  $\gamma$ -IFN and IL-10 responses obtained when PBMC from a given donor were first stimulated in culture with the mitogen Con

A, the recall antigen PPD, or the primary antigen KLH, alone or in combination with purified LMP1. In Figures 7a and 9a, stimuli were also administered in combination with an LMP-1-derived peptide. Cells were rested for seven days, washed to remove the antigens, and added to fresh irradiated autologous PBMC as a source of antigen presenting cells (33). Panel (b) shows the results of restimulating the control cells with each of the three stimuli. Panel (c) shows the results of restimulating the cells originally stimulated in the presence of LMP-1. Panel (d), shown only for Figures 7 and 9, shows the results of restimulating cells originally stimulated in the presence of LMP-1-derived peptide. Results are shown for three EBV-seropositive donors (Figures 7, 8 and 9) and one seronegative donor (Figure 10).

### Examples

**LMP1 induces high levels of IL-10 secretion by PBMC from EBV seropositive but not seronegative donors.**

PBMC from ten EBV seropositive donors were tested for the ability to respond to purified LMP1 with either Th cytokine secretion or proliferation. In all seropositive donors, IL-10 was the predominant cytokine measured, with no significant proliferative, gamma-IFN or IL-4 responses. Figure 1 shows representative results obtained from two seropositive donors. To confirm that the observed responses resulted from previous EBV infection, PBMC from two EBV seronegative donors were tested for responsiveness to the purified LMP1. It can also be seen from Figure 1 that, in these donors, the LMP1 failed to elicit either IL-10 secretion, or significant proliferative and  $\gamma$ -IFN responses. The results in both donor groups are specific to LMP1, since the T-cell mitogen concanavalin A (Con A) and the control recall antigen *Mycobacterium tuberculosis* purified protein derivative (PPD) induced responses dominated by proliferation and  $\gamma$ -IFN production, regardless of EBV serological status.

FIGURE 1. IL-10 secretion by PBMC from EBV seropositive donors in response to LMP1 and Con A. PBMC from two EBV seropositive donors were tested for responsiveness to LMP1, Con A, and PPD. The results are shown as mean  $\pm$  SD. \*p < 0.05.

To further characterize the immune response to LMP1, epitopes that induced IL-10 secretion were mapped by screening PBMC from 20 EBV seropositive healthy donors with a panel of synthetic 20-mer peptides spanning the entire sequence of LMP1. Representative results obtained from one donor (Figure 2) demonstrate that multiple LMP1 peptides induced secretion of high concentrations of IL-10. In contrast, only three peptides induced proliferation, five peptides  $\gamma$ -IFN, two peptides IL-4, and all the latter responses were weak. Similar patterns of responsiveness were found in a total of 20 seropositive donors (summarized in Figure 3). Strikingly, certain peptides commonly elicited IL-10 responses in different donors ( $p=2.2 \cdot 10^{-7}$ , Poisson heterogeneity test), with, for example, peptide 4 (aa 16-35) inducing IL-10 in 80% of the individuals. Furthermore, these dominant IL-10 inducing peptides are clustered within the N terminal half of the protein that is rich in binding motifs for many MHC class II molecules (<http://imtech.res.in/raghava/propred/index.html>; <http://www.csd.abdn.ac.uk/~gjlk/MHC-Thread>) (Sturniolo et al. (1999); Singh and Raghava (2001)).

PBMC from four EBV seronegative donors were also screened with the panel of LMP1 peptides. Reactivity was rare in this group, with totals of only nine IL-10, one  $\gamma$ -IFN, one proliferative and no IL-4 responses. Moreover, all these responses were relatively weak (data not shown).

**Cells responding to LMP1 and LMP1 peptides with IL-10 secretion are CD3<sup>+</sup>CD4<sup>+</sup>.**

The phenotype of the cells responsible for the IL-10 production was determined by flow cytometry, comparing peptide stimulated and unstimulated cultures from four seropositive individuals. Most IL-10 producing cells bore the CD3 marker for T-cells (mean=83.9%, SD=9.4%) and of these the majority were of the CD4<sup>+</sup> helper phenotype (mean=90.6%, SD=8.9%) (Figure 4). Similarly, activated cells, as judged by expression of CD69 and CD71, in the rare cultures proliferating in response to peptides were also CD4<sup>+</sup> (data not shown).

**LMP1 and LMP1 peptides suppress proliferative and gamma-IFN responses by stimulating IL-10 secreting Tr1 cells.**

There is evidence that CD4<sup>+</sup> T-cells biased towards IL-10 secretion, termed Tr1 cells, play an important role in immunoregulation (Groux et al., 1997; Levings and Roncarolo, 2000) and have been shown to inhibit inflammatory responses (Groux et al., 1997; Roncarolo and Levings, 2000). We concluded that the responses to LMP1 and the peptide panel were predominantly mediated by Tr1 cells and sought to confirm that they were capable of mediating suppression. In all ten seropositive donors tested, the addition of LMP1 strongly inhibited proliferative and gamma-IFN responses to the T-cell mitogen Con A, the recall antigen PPD and the primary antigen keyhole limpet hemocyanin (KLH) by 56-99% (Figure 5). The IL-10 responses to LMP1, and the associated inhibition, were dependent on the donor having been infected with EBV, since no such effects were seen when PBMC were obtained from two control seronegative volunteers (Figure 5). Results similar to those obtained with purified LMP1 protein were found when IL-10 inducing LMP1 peptides were added to PBMC cultures from five seropositive donors, with suppression of proliferative and gamma-IFN responses to PPD by 41-99% (Figure 6). In parallel experiments, the peptides also inhibited proliferative and gamma-IFN responses to the mitogen Con A or primary antigen KLH (results not shown). Figure 6 also demonstrates that the inhibitory effect is dependent on IL-10, since, when LMP1 derived peptides that did not elicit this cytokine were added to PPD-stimulated cultures, no suppression was seen. Furthermore, in cultures treated with anti-IL-10 antibody, the LMP1 peptide mediated suppression was reversed by up to 71% (Figure 6).

***In vitro* LMP1 mediated suppression is antigen specific and persistent.**

PBMCs from three seropositive donors and one seronegative donor were first stimulated in culture with the mitogen Con A, the recall antigen PPD, or the primary antigen KLH, alone or in combination with purified LMP1 and in two cases in combination with an IL-10

washed to remove the antigens, and added to fresh irradiated autologous PBMC as a source of antigen presenting cells (Plebanski et al., 1992). Each group of cells was then restimulated with each stimulus. Results are shown in Figures 7 to 10.

5 Cells from seropositive donors (Figures 7, 8 and 9) which were initially exposed to a stimulus in combination with LMP-1 produced significant IL-10 and low IFN-gamma and proliferative responses. When re-stimulated with the same stimulus, in the absence of LMP-1, these cells still failed to proliferate or express IFN-gamma.  
 10 However, they retained the capacity to proliferate and produce IL-10 against other stimuli, showing that the cells had been specifically tolerised to the stimulus originally administered in combination with LMP-1. Similar results were obtained for two of the seropositive donors with LMP-1-derived peptides (peptides 4 and  
 15 18, shown in Figures 7d and 9d respectively).

Cells from the seronegative donor (Figure 10) responded with typical IFN-gamma and proliferative responses to all stimuli regardless of the presence of LMP-1, showing that the induction of tolerance is not an inherent property of the protein, but relies on  
 20 prior exposure of the cells to EBV.

Thus, it can be hypothesised that the Tr1 response to LMP1 deviates T-cells recognizing a bystander antigen to adopt an anergic, IL-10 secreting phenotype. Such induction of anergy specific for other viral antigens that are co-expressed with LMP1 may be important in  
 25 the maintenance of EBV latency.

#### Discussion of Examples

Here a novel mechanism is postulated by which Epstein-Barr virus (EBV), rather than avoiding detection, instead subverts the immune  
 30 response by stimulating regulatory CD4<sup>+</sup> T-cells that secrete the inhibitory cytokine interleukin-10 (IL-10). Such regulatory T-cells are well recognized (1-3) but not known to have a role in viral persistence (4-6). Human peripheral blood mononuclear cells (PBMC) from all EBV seropositive, but not seronegative, donors  
 35 responded to both purified latent membrane protein 1 (LMP1) and the

corresponding immunodominant peptides with high levels of IL-10 secretion by CD4<sup>+</sup> T-cells. These IL-10 responses, characteristic of T regulatory 1 (Tr1) cells, coincided with inhibition of T-cell proliferation and  $\gamma$ -interferon ( $\gamma$ -IFN) secretion induced by both mitogen and recall antigen. The ability of this viral antigen to deviate the immune response towards tolerance is likely to be important in maintaining latency and EBV associated tumors.

This study was prompted by the lack of a protective immune response against LMP1 in individuals with latent EBV infection. The main conclusion from our data is that LMP1 is recognized by the immune system, but that this response is dominated by the induction of high levels of IL-10 secretion by cells with a Tr1 phenotype (Levings and Roncarolo, 2000). Furthermore, this IL-10 response was able to suppress both proliferative and  $\gamma$ -IFN responses against other antigens and polyclonal stimuli, and therefore would be expected to prevent the development of protective Th1 and cytotoxic immunity against LMP1 (Fiorentino et al., 1991). Indeed, such IL-10 secretion is also likely to anergise Th1 responses to other EBV proteins co-expressed both in latent infection and associated tumors. Our demonstration of Tr1 activation provides a mechanism for the previously reported observation that recombinant LMP1 inhibits immune functions including mitogen, antigen and CD3/CD28 stimulated T-cell activation; natural killer cell cytotoxicity; and antigen-induced gamma-IFN secretion (Dukers et al., 2000). One peptide from LMP1, included within the sequence of peptide 7 (aa 31-50) from our panel, was reported (Dukers et al., 2000) to replicate these inhibitory properties, and is identified here as one of many effective Tr1 IL-10 inducers.

A possible explanation for the propensity of LMP1 to elicit IL-10 production by Tr1 cells is that the establishment and maintenance of a suppressive response to LMP1 results from IL-10 'conditioning'. Activation of CD4<sup>+</sup> T-cells in the presence of IL-10 leads to the generation of Tr1 cells (Groux et al., 1997). EBV

lytic cycle infection (Hayes et al., 1999). Thus, we propose that, during the development of the immune response to EBV, the presence of vIL-10 deviates the differentiation of LMP1 specific Th cells to favor IL-10 secreting Tr1 cells, a bias which then becomes self-perpetuating. The finding that the regulatory response to LMP1 is limited to EBV seropositive, but not seronegative, donors strongly supports this second explanation.

Numerous methods used by pathogens to avoid clearance by the immune system have been described, dependent on escape, resistance or counterattack (Xu et al., 2001). The induction of a Tr1 response to EBV LMP1 represents a further mechanism of immune evasion. A similar regulatory mechanism that subverts, rather than avoids, immune detection may well be exploited by other pathogens with the ability to maintain chronic infections. This is especially likely for those viruses, such as cytomegalovirus, that also encode a homologue of IL-10 with potent immunosuppressive effects (Spencer et al., 2002), which may also induce a regulatory Tr1 type immune response. The design of strategies to overcome such Tr1 responses should provide an innovative approach to the development of vaccines to prevent or treat EBV associated tumors. Conversely, it may be possible to exploit therapeutically such specific induction of bystander anergy to inhibit pathogenic responses in immune-mediated diseases.

## Experimental Procedures

### **Donors**

Blood samples were obtained by venepuncture from a group of healthy volunteers. The donors were classified as EBV seropositive or seronegative by an ELISA for serum anti-EBNA1 IgG, with negative results confirmed by immunofluorescence staining for IgG and IgM anti-viral capsid antibody.

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### **Antigens and Mitogen**



LMP1 was immunopurified from lysed EBV transformed B cells using the anti-LMP1 antibody CS1-4 (Novocastra Laboratories) conjugated to anti-mouse IgG<sub>1</sub> coated magnetic beads (Biomag, PerSeptive Biosystems).

A panel of 76 20-mer peptides, with 15 amino acid overlaps, was synthesised (Department of Biochemistry, University of Birmingham, UK or University of Bristol, UK), spanning the entire length of the 63kD EBV LMP1, as determined from the prototype B-cell-derived gene (B95.8) sequence (Hayes et al., 1999). All peptides were used to stimulate cultures at 15µg/ml, although, as in previous mapping studies (Stott et al., 2000), responses were similar over a wide range of concentrations (4-50µg/ml).

Peptide sequences are as follows:

|     |                        |     |                       |
|-----|------------------------|-----|-----------------------|
| P1  | MEHDLERGP              | P39 | HSDEHHHDDSLPHPQQATDD  |
| P2  | ERGPPGPRRPPRGPPPLSSSL  | P40 | HHDDSLPHPQQATDDSGHES  |
| P3  | GPRRPPRGPPPLSSSLGLALL  | P41 | LPHPQQATDDSGHESDSNSN  |
| P4  | PRGPPPLSSSLGLALLLLLLA  | P42 | QATDDSGHESDSNSNEGRHH  |
| P5  | LSSSLGLALLLLLLALLFWL   | P43 | SGHESDSNSNEGRHHLLVSG  |
| P6  | GLALLLLLLALLFWLYIVMS   | P44 | DSNSNEGRHHLLVSGAGDGP  |
| P7  | LLLLALLFWLYIVMSDWTGG   | P45 | EGRHHLLVSGAGDGPPLCSQ  |
| P8  | LLFWLYIVMSDWTGGALLVL   | P46 | LLVSGAGDGPPLCSQNLGAP  |
| P9  | YIVMSDWTGGALLVLYSFAL   | P47 | AGDGPPLCSQNLGAPGGGPD  |
| P10 | DWTGGALLVLYSFALMLIII   | P48 | PLCSQNLGAPGGGPDNGPQD  |
| P11 | ALLVLYSFALMLIIIIILIIIF | P49 | NLGAPGGGPDNGPQDPDNTD  |
| P12 | YSFALMLIIIIILIIIFIFRRD | P50 | GGGPDNGPQDPDNTDDNGPQ  |
| P13 | MLIIIIILIIIFIFRRDLLCPL | P51 | NGPQDPDNTDDNGPQDPDNT  |
| P14 | ILIIIFIFRRDLLCPLGALCI  | P52 | PDNTDDNGPQDPDNTDDNGP  |
| P15 | IFRRDLLCPLGALCILLMI    | P53 | DNGPQDPDNTDDNGPHDPLP  |
| P16 | LLCPLGALCILLMITLLLI    | P54 | DPDNTDDNGPHDPLPQDPDN  |
| P17 | GALCILLMITLLLIALWNL    | P55 | DDNGPHDPLPQDPDNTDDNG  |
| P18 | LLLMITLLLIALWNLHGQAL   | P56 | HDPLPQDPDNTDDNGPQDPD  |
| P19 | TLLLIALWNLHGQALFLGIV   | P57 | QDPDNTDDNGPQDPDNTDDN  |
| P20 | ALWNLHGQALFLGIVLFIFG   | P58 | TDDNGPQDPDNTDDNGPHDP  |
| P21 | HGQALFLGIVLFIFGCLLVL   | P59 | PQDPDNTDDNGPHDPLPHSP  |
| P22 | FLGIVLFIFGCLLVLGIIY    | P60 | NTDDNGPHDPLPHSPSDSAG  |
| P23 | LFIFGCLLVLGIIYLLLEML   | P61 | GPHDPLPHSPSDSAGNDGGP  |
| P24 | CLLVLGIIYLLLEMLWRLGA   | P62 | LPHSPSDSAGNDGGPPQLTE  |
| P25 | GIWIYLLLEMLWRLGATIWQL  | P63 | SSGSGGDDDDPHGVPQLSYYD |
| P26 | LLEMLWRLGATIWQLLAFFL   | P64 | SDSAGNDGGPPQLTEEVENK  |
| P27 | WRLGATIWQLLAFFLAFFLD   | P65 | NDGGPPQLTEEVENKGGDQG  |
| P28 | TIWQLLAFFLAFFLDLILLI   | P66 | PQLTEEVENKGGDQGGPPLMT |
| P29 | TEFFLAFFLDLILLIILII    | P67 | EVENKGGDQGGPPLMTDGGG  |
| P30 | TEFFLAFFLDLILLIILII    |     |                       |
| P31 | TEFFLAFFLDLILLIILII    |     |                       |
| P32 | TEFFLAFFLDLILLIILII    |     |                       |
| P33 | TEFFLAFFLDLILLIILII    |     |                       |
| P34 | TEFFLAFFLDLILLIILII    |     |                       |
| P35 | TEFFLAFFLDLILLIILII    |     |                       |
| P36 | TEFFLAFFLDLILLIILII    |     |                       |
| P37 | TEFFLAFFLDLILLIILII    |     |                       |
| P38 | TEFFLAFFLDLILLIILII    |     |                       |

P33 QQNWWTLLVDLLWLLLFLAI  
 P34 TLLVDLLWLLLFLAILIWMY  
 P35 LLWLLLFLAILIWMYYHGQR  
 P36 LFLAILIWMYYHGQRHSDEH  
 P37 LIWMYYHGQRHSDEHHHDDS  
 P38 YHGQRHSDEHHHDDSLPHPQ

P71 HSHDSGHGGGDPHLPTLLLG  
 P72 GHGGGDPHLPTLLLGSSSGS  
 P73 DPHLPTLLLGSSSGSGDDDD  
 P74 TLLLGSSSGSGDDDDDPHGPV  
 P75 LFLAILIWMYYHGQRHSDEH

A similar panel of peptides was prepared from LMP2 having sequences as follows:

P1' MGSLEMVPMGAGPPSPGGDP  
 P2' MVMGAGPPSPGGDPDGYDG  
 P3' AGPPSPGGDPDGYDGGNNSQ  
 P4' PGGDPDGYDGGNNSQYPSAS  
 P5' DGYDGGMNSQYPSASGSSGN  
 P6' GNSQYPSASGSSGNTPTPP  
 P7' YPSASGSSGNTPTPPMDEER  
 P8' GSSGNTPTPPNDEERESNEE  
 P9' TPTPPNDEERESNEEPPPPY  
 P10' NDEERESNEEPPPPYEDPYW  
 P11' RESNEEPPPPYEDYWGNGD  
 P12' EPPPPYEDPYWGNGDRHSDY  
 P13' YEDPYWGNGDRHSDYQPLGT  
 P14' WGNGDRHSDYQPLGTQDQSL  
 P15' RHSDYQPLGTQDQSLYLGLQ  
 P16' QPLGTQDQSLYLGLQHDGND  
 P17' QDQSLYLGLQHDGNDGLPPP  
 P18' LGLQHDGNDGLPPPYSRDP  
 P19' DGNDGLPPPYSRDDSSQH  
 P20' LPPPPYSRDDSSQHIYEEA  
 P21' PPYSRDDSSQHIYEEAGRG  
 P22' RDDSSQHIYEEAGRGSMNPV  
 P23' QHIYEEAGRGSMNPVCLPVI  
 P24' EAGRGSMMPVCLPVIVAPYL  
 P25' SMNPVCLPVIVAPYLFWLAA  
 P26' CLPVIVAPYLFWLAAIAASC  
 P27' VAPYLFWLAAIAASCFTASV  
 P28' FWLAAIAASCFTASVSTVVT  
 P29' IAASCFTASVSTVVTATGLA  
 P30' FTASVSTVVTATGLALSLLL  
 P31' STVVTATGLALSLLLLAAVA  
 P32' ATGLALSLLLLAAVASSYAA  
 P33' LLLLLAAVASSYAAAQRKL  
 P34' LAAVASSYAAAQRKLLTPVT  
 P35' SSYAAAQRKLLTPVTVLTAV  
 P36' AQRKLLTPVTVLTAVVTFFA  
 P37' LTPVTVLTAVVTFFAICLTW  
 P38' VLTAVVTFFAICLTWRIEDP  
 P39' VTFFAICLTWRIEDPPFNSL  
 P40' ICLTWRIEDPPFNSLLFALL  
 P41' RIEDPPFNSLLFALLAAAGG  
 P42' PFNSLLFALLAAAGGLQGIY  
 P43' LFALLAAAGGLQGIYVLVML  
 P44' AAAGGLQGIYVLVMLVLLIL

P49' WRRITVCGGIMFLACVLVLI  
 P50' VCGGIMFLACVLVLIVDAVL  
 P51' MFLACVLVLIVDAVLQLSPL  
 P52' VLVVLIVDAVLQLSPLLGAVT  
 P53' VDAVLQLSPLLGAVTVVSMT  
 P54' QLSPLLGAVTVVSMTLLLLA  
 P55' LGAVTVVSMTLLLLAFVLWL  
 P56' VVSMTLLLLAFVLWLSSPGG  
 P57' LLLLLAFVLWLSSPGGLGTLG  
 P58' FVLWLSSPGGLGTLGAALLT  
 P59' SSPGGLGTLGAALLTLAAAL  
 P60' LGTLGAALLTLAAALALLAS  
 P61' AALLTLAAALALLASLILGT  
 P62' LAAALALLASLILGTNLTT  
 P63' ALLASLILGTNLTTMFLLM  
 P64' LILGTNLTTMFLMLLWTL  
 P65' LNLTTMFLMLLWTLVLLI  
 P66' MFLMLLWTLVLLICSSCS  
 P67' LLWTLVLLICSSCSCPLS  
 P68' VLLICSSCSCPLSKILLA  
 P69' CSSCSCPLSKILLARFLY  
 P70' SCPLSKILLARFLYALALL  
 P71' KILLARFLYALALLLLASA  
 P72' RLFLYALALLLLASALIAGG  
 P73' ALALLLLASALIAGGSILQT  
 P74' LLASALIAGGSILQTNFKSL  
 P75' LIAGGSILQTNFKSLSSSTEF  
 P76' SILQTNFKSLSSSTEFIPNLF  
 P77' NFKSLSSSTEFIPNLFMLLL  
 P78' SSTEIPNLFMLLLIVAGI  
 P79' IPNLFMLLLIVAGILFIL  
 P80' CMLLLIVAGILFILAILTEW  
 P81' IVAGILFILAILTEWGSGNR  
 P82' LFILAILTEWGSGNRITYGPV  
 P83' ILTEWGSGNRITYGPVFMCLG  
 P84' GSGNRITYGPVFMCLGGLLTM  
 P85' FMCLGGLLTMVAGAVWLTVM  
 P86' GLLTMVAGAVWLTVMSTLL  
 P87' VAGAVWLTVMSTLLSAWIL  
 P88' WLTVMSTLLSAWILTAGFL  
 P89' SNTLLSAWILTAGFLIFLIG  
 P90' SAWILTAGFLIFLIGFALFG  
 P91' TAGFLIFLIGFALFGVIRCC  
 P92' IFLIGFALFGVIRCCRYCCY

|      |                      |      |                      |
|------|----------------------|------|----------------------|
| P45' | LQGIYVLVMLVLLILAYRRR | P93' | FALFGVIRCCRYCCYYCLTL |
| P46' | VLVMLVLLILAYRRRWRLT  | P94' | VIRCCRYCCYYCLTLESEER |
| P47' | VLLILAYRRRWRLTVCGGI  | P95' | RYCCYYCLTLESEERPPTY  |
| P48' | AYRRRWRLTVCGGIMFLAC  | P96' | YYCLTLESEERPPTYRNTV  |

The control antigen mycobacterial PPD (Statens Seruminstitut), the T-cell mitogen Con A (Sigma), and the primary antigen keyhole limpet hemocyanin (KLH) (Calbiochem) were each used to stimulate cultures at 10µg/ml. PPD readily provokes recall T-cell responses *in vitro* (Plebanski et al., 1992), since most UK citizens have been immunised with Bacille Calmette-Guérin (BCG).

#### Cell Proliferation and Cytokine Assays

As described elsewhere (Stott et al., 2000), PBMC were separated from fresh blood samples by density gradient centrifugation and cultured in 1ml volumes at a concentration of  $1.25 \times 10^6$  cells/ml. Cellular proliferation was estimated from the incorporation of  $^3\text{H}$ -thymidine in triplicate 100µl aliquots taken from the wells five days after antigen stimulation, when recall T-cell responses are maximal (Plebanski et al., 1992). Proliferation results are presented as the mean counts per minute (CPM)  $\pm$  SD of the triplicate samples, or as stimulation index (SI), expressing the ratio of mean CPM in stimulated versus unstimulated control cultures. An  $\text{SI} > 3$  with  $\text{CPM} > 1000$  is interpreted as representing a significant positive response (Devereux et al., 2000). Production of the Th cytokines  $\gamma$ -IFN, IL-4 and IL-10 was assessed in duplicate 100µl aliquots taken five days after stimulation of the cultures, using a sensitive cellular ELISA (Devereux et al., 2000). Cytokine responses over twice the production in unstimulated cultures were considered positive (Devereux et al., 2000).

#### Characterisation of Responding Cells

The phenotypes of cultured cells that proliferate or secrete cytokine in response to antigen were determined by flow cytometry.

Mononuclear cells were taken from responding cultures and stained

isothiocyanate, with anti-CD25 phycoerythrin-cyanin 5.1 (all Beckman Coulter) in some experiments. Activated cells in proliferating cultures were identified using anti-CD71-phycoerythrin (PE) or anti-CD69-PE (both Beckman Coulter). Cells synthesising IL-10 were labelled by incubating with anti-IL-10-PE (Pharmingen) after inhibition of protein secretion with Brefeldin A (Sigma) and permeabilisation with Intraprep™ (Beckman Coulter). Stained cells were analysed using an EPICS XL cytometer (Beckman Coulter) and Expo v2 analysis software (Applied Cytometry Systems).

10 All documents cited herein are hereby incorporated by reference insofar as is required for the skilled person to carry out the invention.

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CLAIMS:

1. A method for assessing the tolerogenicity of a test peptide sequence from an infectious agent, comprising the steps of:

(i) contacting a cell population with said test peptide sequence,  
(ii) determining whether IL-10 expression in said cell population is increased, and optionally

(iii) correlating the result of step (ii) with the tolerogenicity of the sequence,

wherein said cell population comprises mononuclear leukocytes from a donor previously infected by said infectious agent.

2. A method according to claim 1, wherein said cell population comprises at least one type of antigen presenting cell.

3. A method according to claim 1 or claim 2, wherein said mononuclear leukocytes comprise at least T lymphocytes, B lymphocytes, natural killer (NK) cells, monocytes, macrophages or dendritic cells.

4. A method according to claim 3, wherein said mononuclear leukocytes comprise at least CD4<sup>+</sup> T lymphocytes.

5. A method according to claim 4, wherein said mononuclear leukocytes further comprise at least one type of antigen presenting cell.

6. A method according to any one of claims 1 to 5, further comprising the steps of:

(i) (a) contacting a similar cell population from a donor not previously infected by said infectious agent with said test peptide sequence; and

(ii) (a) determining whether IL-10 expression in said cell population is increased,

and optionally

(ii) (b) comparing the results from step (ii) with the results from step (ii) (a).

7. A method according to any one of claims 1 to 6, wherein the infectious agent is a virus.

8. A method according to claim 7, wherein the virus is a herpesvirus encoding a viral IL-10 homologue

9. A method according to claim 8, wherein the virus is EBV.

10. A method according to claim 9, wherein the test peptide sequence is derived from EBV LMP1 protein or LMP2 protein.

11. A method according to claim 10, wherein the test or tolerogenic peptide sequence comprises one or more of the sequences P1 to P75 or P1' to P96'.

12. A method for assessing the tolerogenicity of a test peptide sequence from an infectious agent towards a target antigen, comprising the steps of:

(i) contacting a cell population with (a) said test peptide sequence and (b) a target antigen, to make a test composition, and

(ii) re-contacting the cell population from said test composition with said target antigen.

wherein said cell population comprises mononuclear leukocytes from a donor previously infected by said infectious agent.

13. A method according to claim 12, further comprising the steps of:

5 (iii) assessing the response of said cell population to said target antigen, and optionally

(iv) correlating the result of step (iii) with the tolerogenicity of the test peptide sequence.

10 14. A method according to claim 13, wherein step (iii) comprises assessment of cell proliferation or expression of IL-4, IL-2, IL-12 or gamma-IFN.

15 15. A method according to claims 12 to 14, further comprising the step of adding fresh antigen presenting cells prior to step (ii).

20 16. A method according to any one of claims 12 to 15, further comprising the step of contacting the cell population with a confirmatory antigen unrelated to the test sequence or the target antigen.

17. A method according to any one of claims 12 to 16, wherein the infectious agent is a virus.

25 18. A method according to claim 17, wherein the virus is a herpesvirus encoding a viral IL-10 homologue

19. A method according to claim 18, wherein the virus is EBV.

30 20. A method according to claim 19, wherein the test peptide sequence is derived from EBV LMP1 protein or LMP2 protein.

35 21. A method according to claim 20, wherein the test or tolerogenic peptide sequence comprises one or more of the sequences P1 to P75 or P1' to P96'.

22. A method for assessing the tolerogenicity of a test peptide sequence, comprising the steps of:

5 (i) contacting a first cell population with said test peptide sequence,

(ii) contacting a second cell population with a control peptide sequence

0 (iii) determining whether IL-10 expression in each said cell population is increased, and optionally

(iv) correlating the result of step (iii) with the tolerogenicity of the test peptide sequence,

5 wherein each said cell population comprises mononuclear leukocytes from a donor previously infected by an infectious agent, and said control peptide sequence is derived from said infectious agent.

0 23. A method according to claim 22 wherein said control peptide sequence has previously been identified to induce IL-10 expression in a cell population comprising mononuclear leukocytes from a donor previously infected by said infectious agent.

5 24. A method according to claim 21 or claim 22, wherein said first and second cell populations are derived from the same donor.

25. A method according to claim 24, wherein said first and second cell populations comprise a T cell clone capable of proliferating  
0 in response to the control peptide.

26. A method according to any one of claims 22 to 25, wherein said infectious agent is EBV.

5 27. A method according to claim 26, wherein said control peptide is derived from IMF1 or IMF2.

28. A method according to claim 27, wherein said control peptide comprises one or more of P1 to P75 and P1' to P96'.

29. A method of tolerising a cell population to a target antigen,  
comprising contacting said cell population with

(a) a tolerogenic peptide sequence from an infectious agent, or a nucleic acid encoding said test peptide sequence, such that said test peptide sequence is expressed in said cell population; and

(b) the target antigen, or a nucleic acid encoding said test peptide sequence, such that said test peptide sequence is expressed in said cell population;

wherein said cell population comprises mononuclear leukocytes from a subject seropositive for said infectious agent.

30. A method according to claim 29, comprising the steps of contacting a population of antigen presenting cells with said tolerogenic peptide sequence and said target antigen, and subsequently contacting said cell population with said population of antigen presenting cells.

31. A method according to claim 29 or claim 30, wherein said mononuclear leukocytes are contacted with said tolerogenic peptide sequence and said target antigen *in vitro*.

32. A method according to claim 31, wherein said cell population or a subset thereof is re-administered to said subject after contacting with said tolerogenic peptide sequence and said target antigen.

33. A method according to claim 30, wherein said population of antigen presenting cells is contacted with said tolerogenic peptide sequence and said target antigen *in vitro* and said cell population is contacted with said population of antigen presenting cells *in vivo*.



34. A method according to claim 29, wherein said tolerogenic peptide sequence and said target antigen are administered directly to said subject.

35. A method according to any one of claims 29 to 34, wherein the infectious agent is a virus.

36. A method according to claim 35, wherein the virus is a herpesvirus encoding a viral IL-10 homologue.

37. A method according to claim 36, wherein the virus is EBV.

38. A method according to claim 37, wherein the tolerogenic peptide sequence is derived from EBV LMP1 protein or LMP2 protein.

39. A method according to claim 38, wherein the tolerogenic peptide sequence comprises one or more of the sequences P1 to P75 or P1' to P96'.

40. A pharmaceutical composition, for tolerising a subject against a target antigen, comprising a tolerogenic peptide sequence from an infectious agent, or a nucleic acid encoding a tolerogenic peptide sequence from an infectious agent, in admixture with a pharmaceutically acceptable carrier.

41. A pharmaceutical composition according to claim 40, wherein the tolerogenic peptide sequence is derived from EBV.

42. A pharmaceutical composition according to claim 41, wherein the tolerogenic peptide sequence is derived from LMP1 or LMP2.

43. A pharmaceutical composition according to claim 42, wherein the tolerogenic peptide sequence comprises one or more of the peptide sequences P1 to P75 or P1' to P96'.

44. A pharmaceutical composition according to any one of claims 40 to 43, further comprising said target antigen.

5 45. A pharmaceutical composition according to any one of claims 41 to 44, for administration to an individual previously infected with EBV.

10 46. Use of EBV LMP1, LMP2, or a fragment or mimetic thereof, or a nucleic acid encoding the same, in the preparation of a medicament for the prophylaxis or treatment of a condition mediated by an immune response directed against a target antigen.

15 47. Use according to claim 46, wherein the medicament is formulated for administration in conjunction with the target antigen or a nucleic acid encoding the target antigen.

20 48. Use according to claim 46 or 47, wherein the medicament comprises the target antigen or a nucleic acid encoding the target antigen.

25 49. Use according to any one of claims 46 to 48, wherein the condition is type I diabetes mellitus, coeliac disease, multiple sclerosis, rheumatoid arthritis, systemic lupus erythaematosus, myaesthesia gravis, autoimmune haemolytic anaemia, thrombocytopenia, an atopic response or allergy, or a response to a therapeutic product.

30 50. Use according to any one of claims 46 to 48 wherein the target antigen is a cell.

51. Use according to claim 50 wherein the cell is for transplantation.

35 52. Use according to claim 51 in combination with claim 48 wherein the cell comprises nucleic acid encoding the tolerogenic peptide sequence.

53. Use according to any one of claims 46 to 52 wherein the medicament is formulated for administration to an individual previously infected with EBV.

5 54. A peptide having the sequence of any one of P1 to P75 and P1' to P96'.

10 55. A peptide according to claim 54, having the sequence of any one of P2, P4, P5, P6, P7, P8, P9, P10, P12, P13, P14, P15, P16, P17, P18, P20, P22, P23, P24, P25, P26, P27, P29, P30, P32, P34, P35, P39, P68, P71, P72.

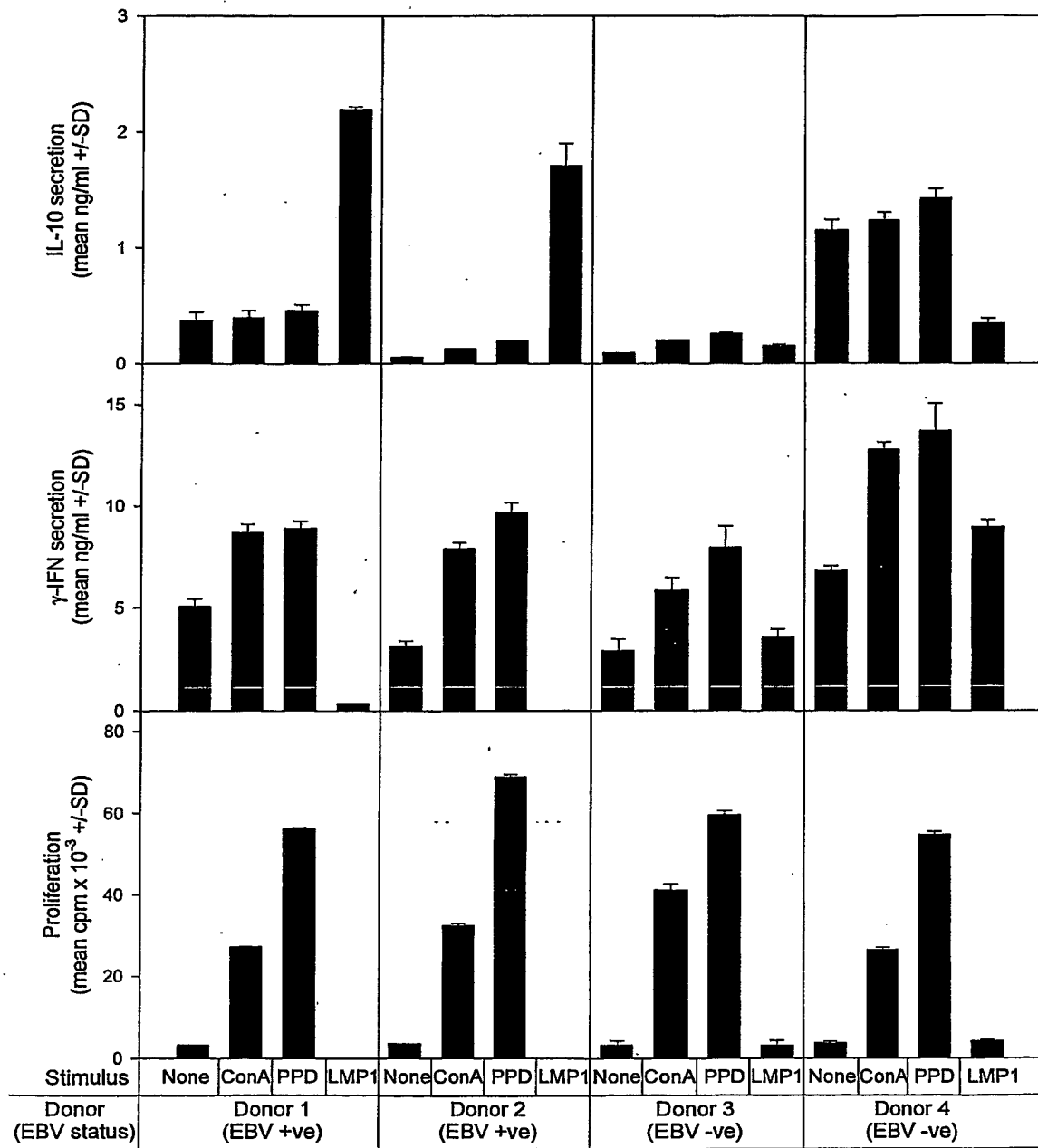


Figure 1

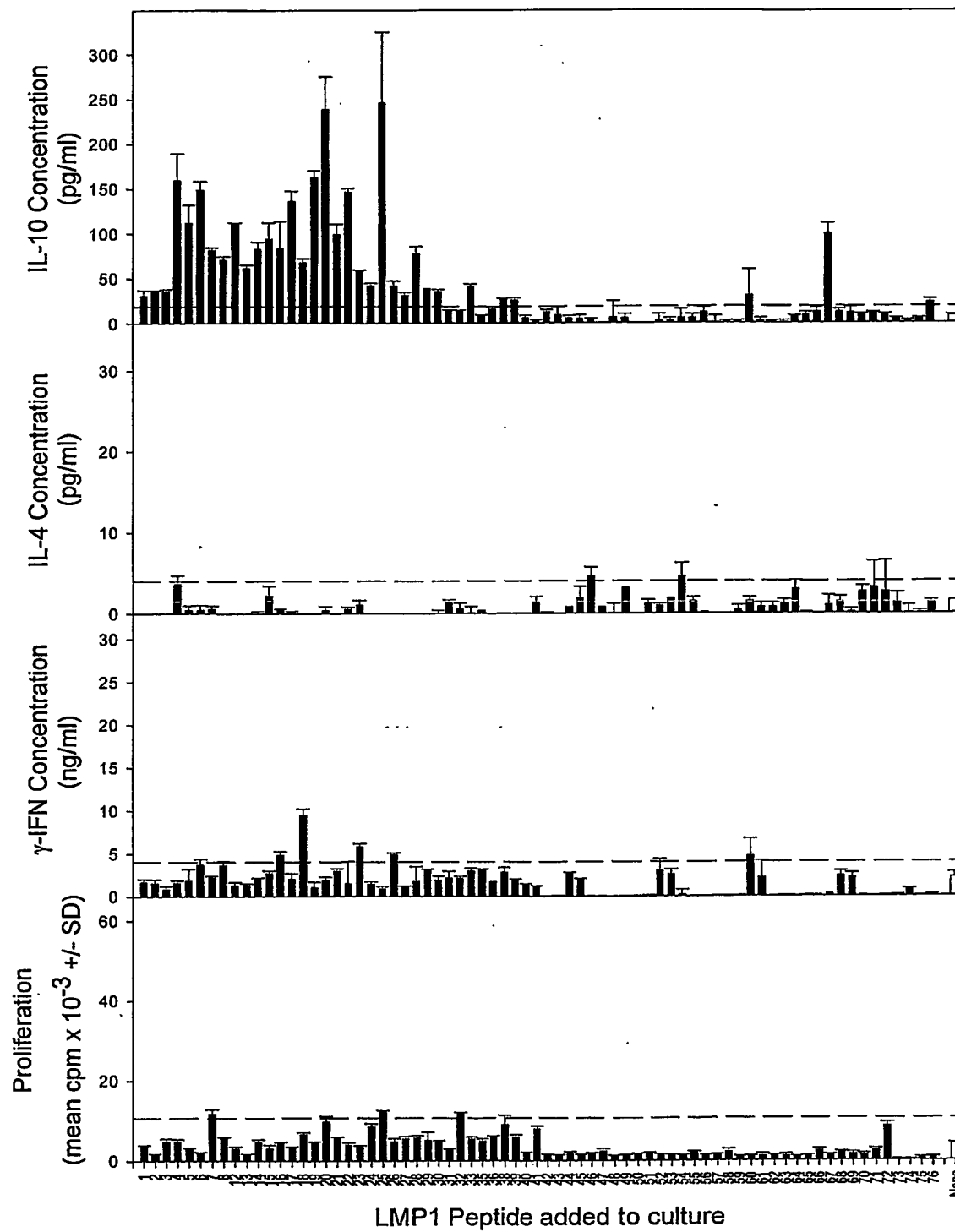


Figure 2

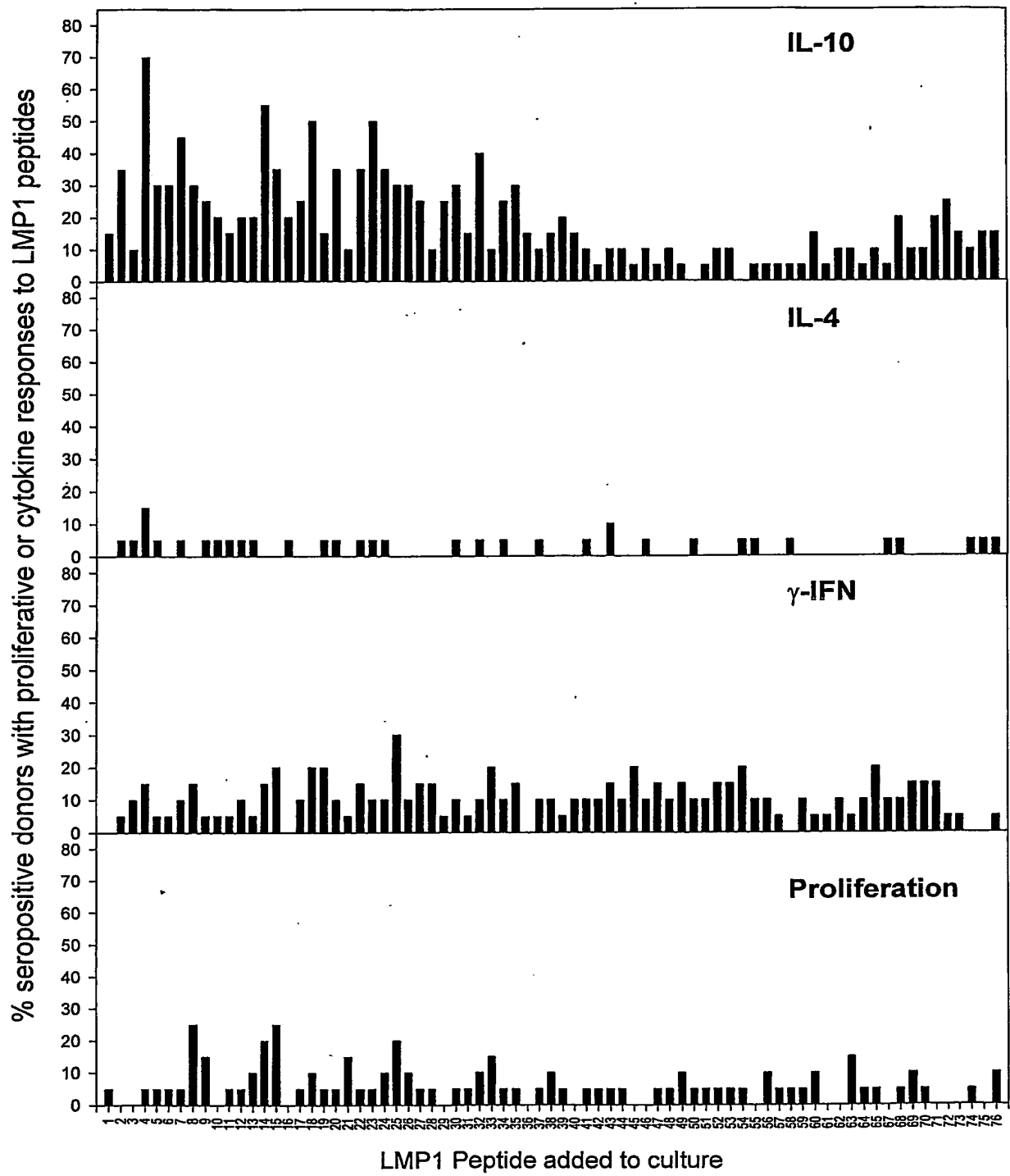


Figure 3

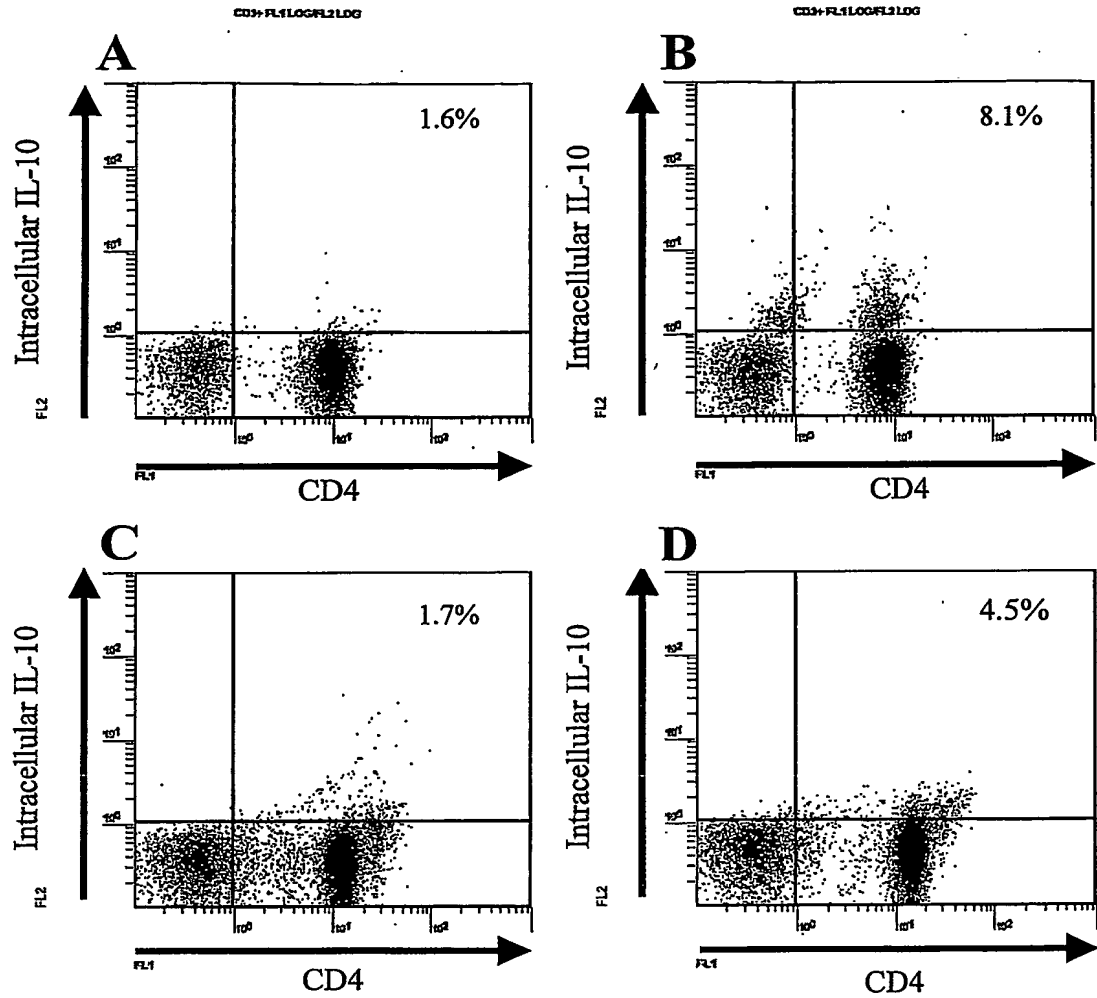


Figure 4

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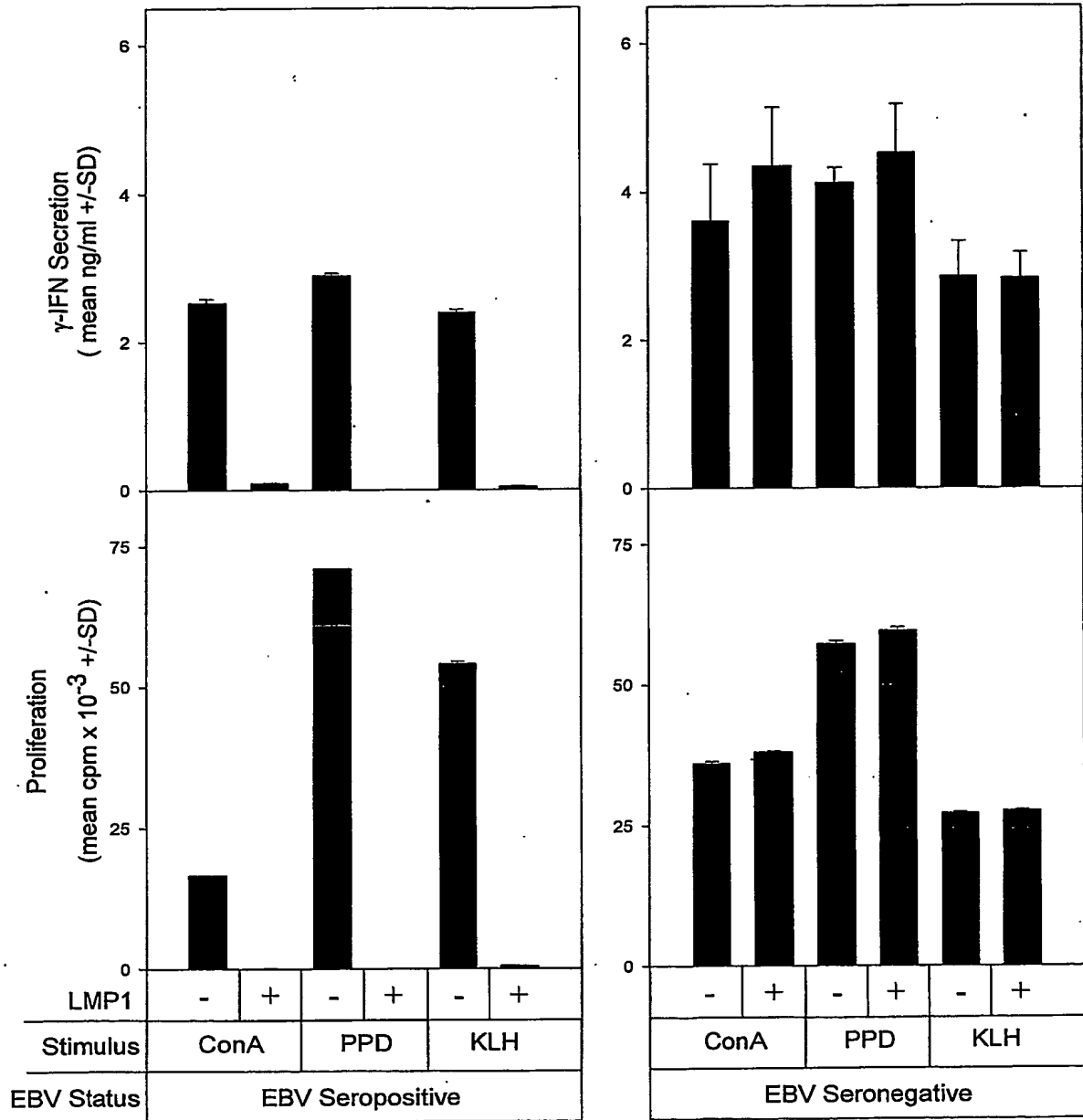


Figure 5



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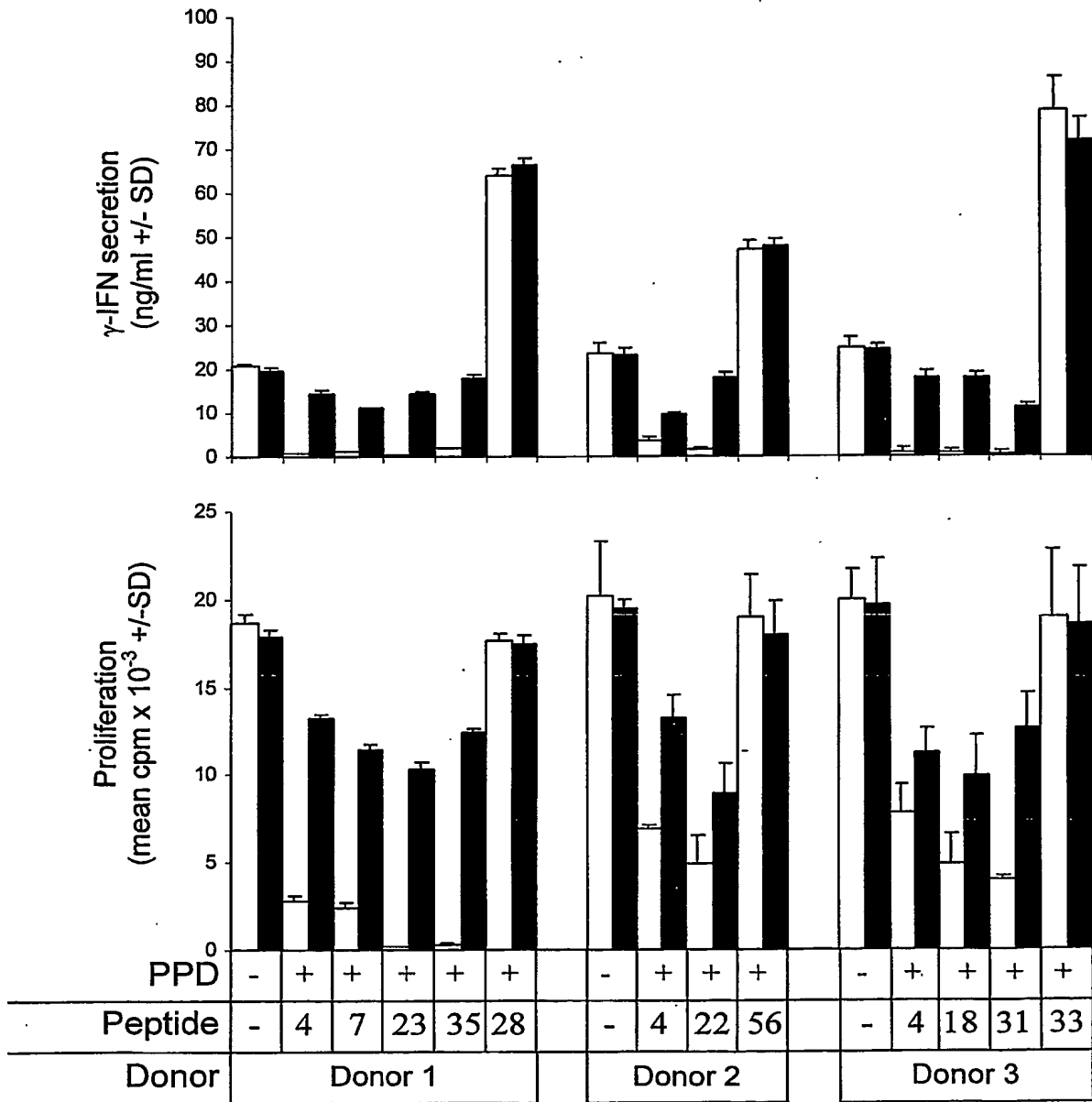


Figure 6

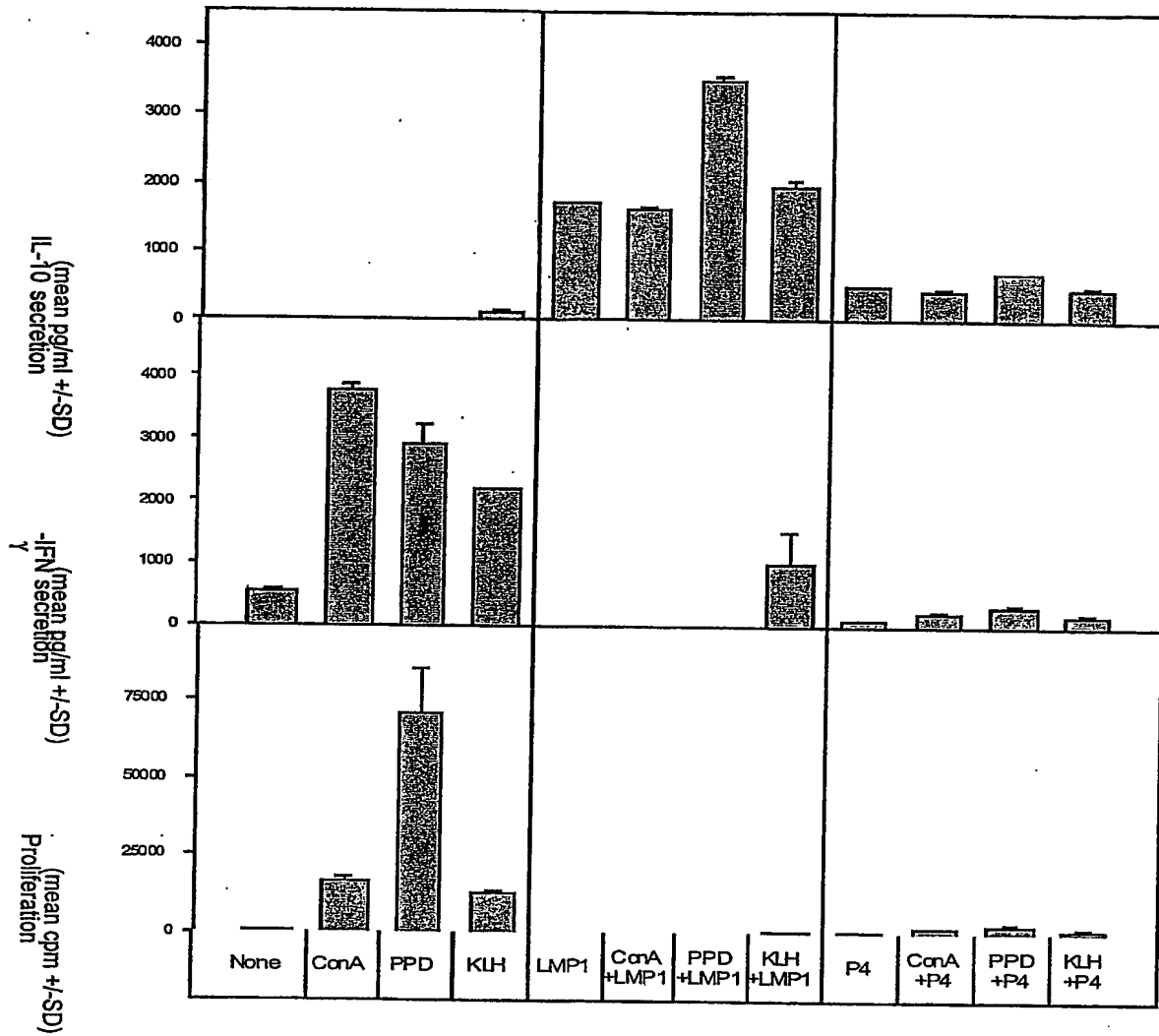


Figure 7a

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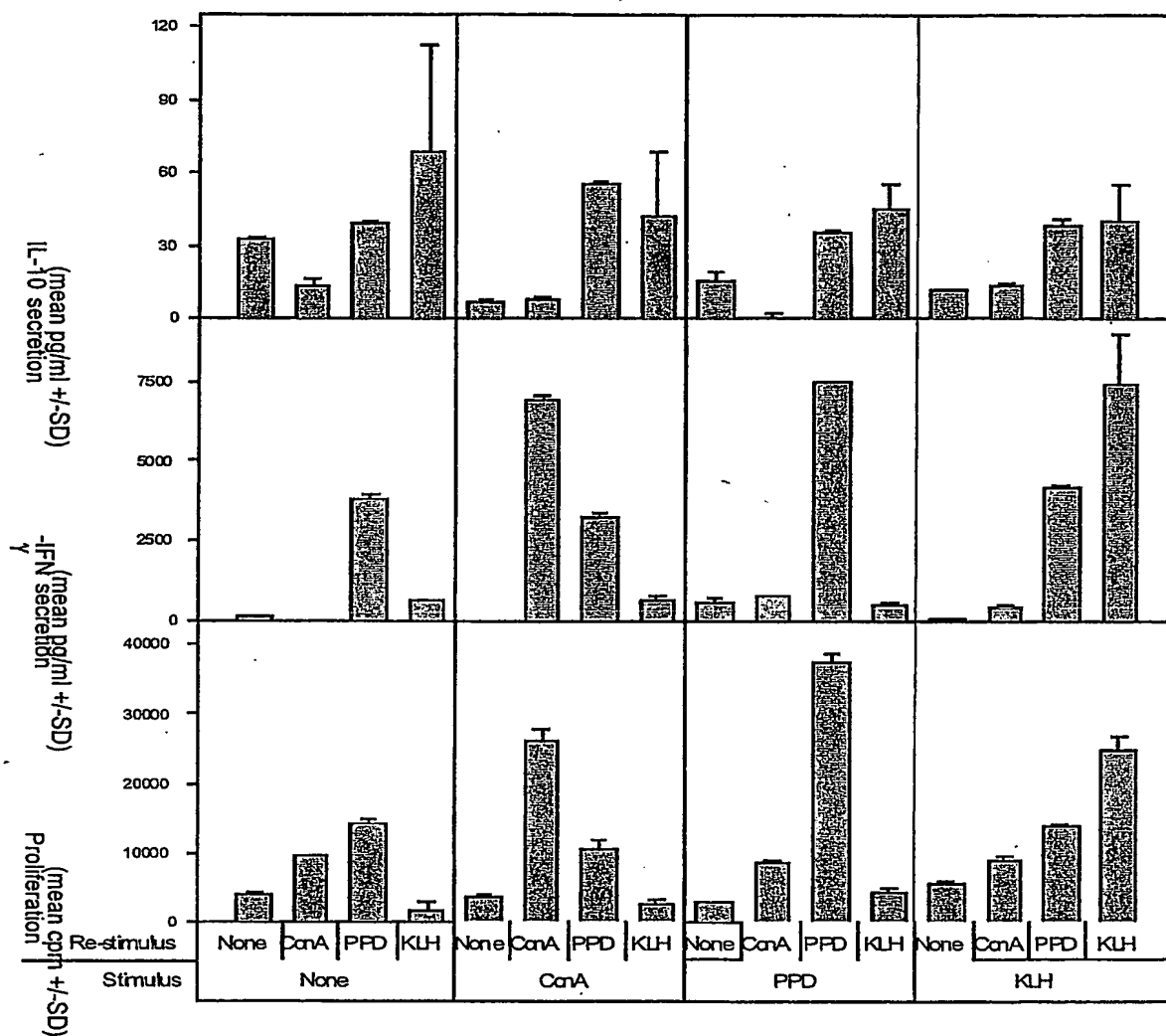


Figure 7b

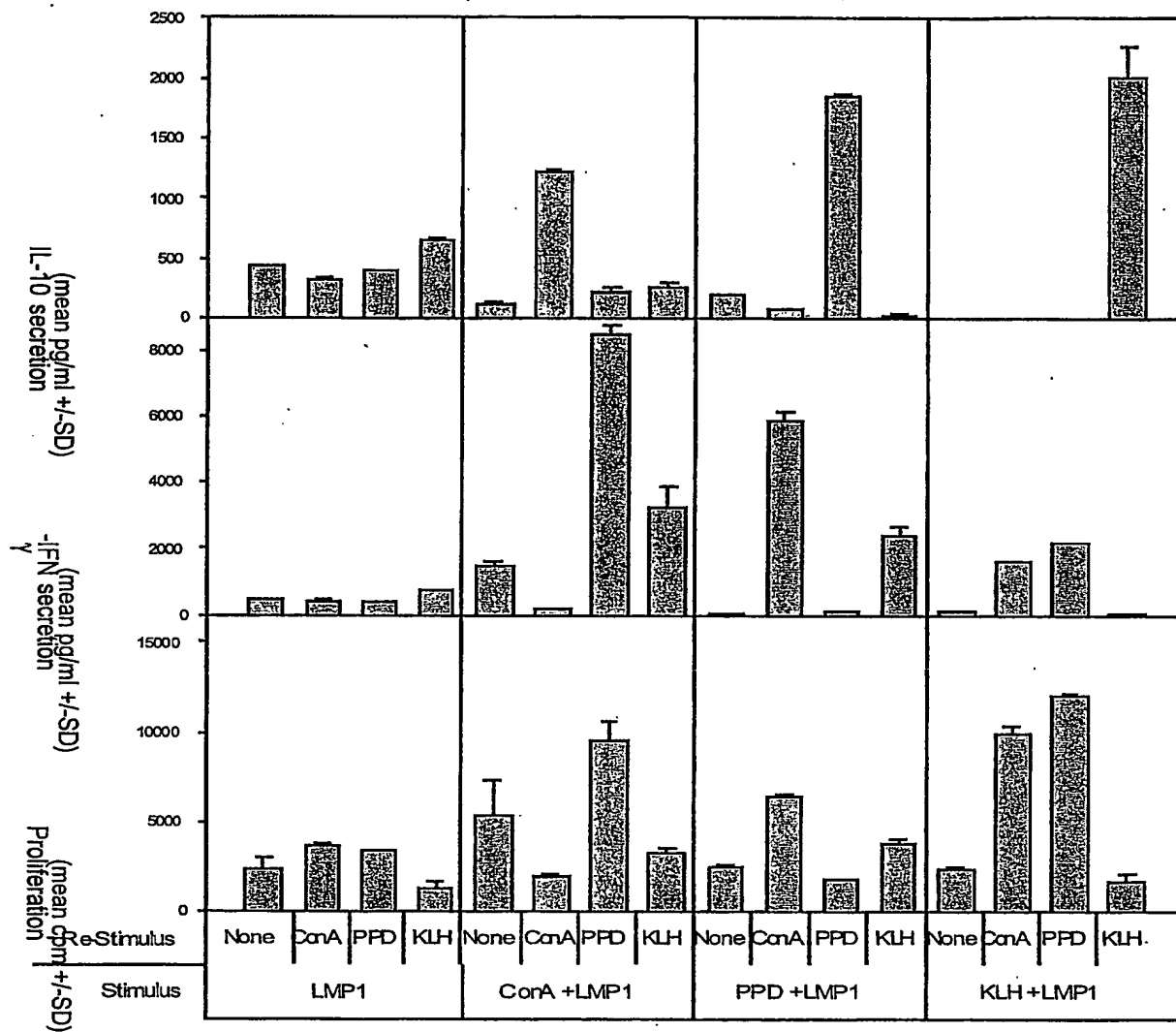


Figure 7c

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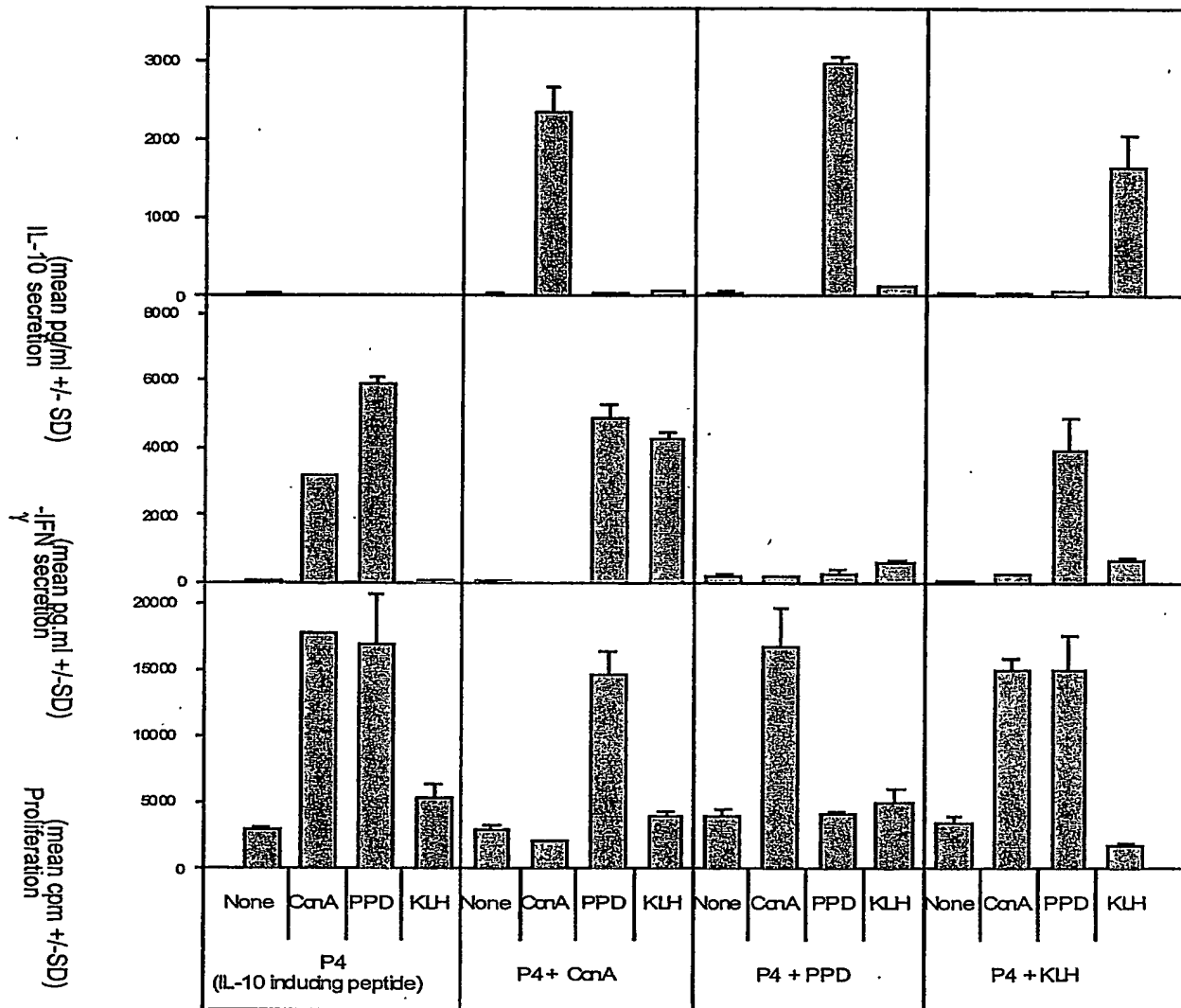


Figure 7d

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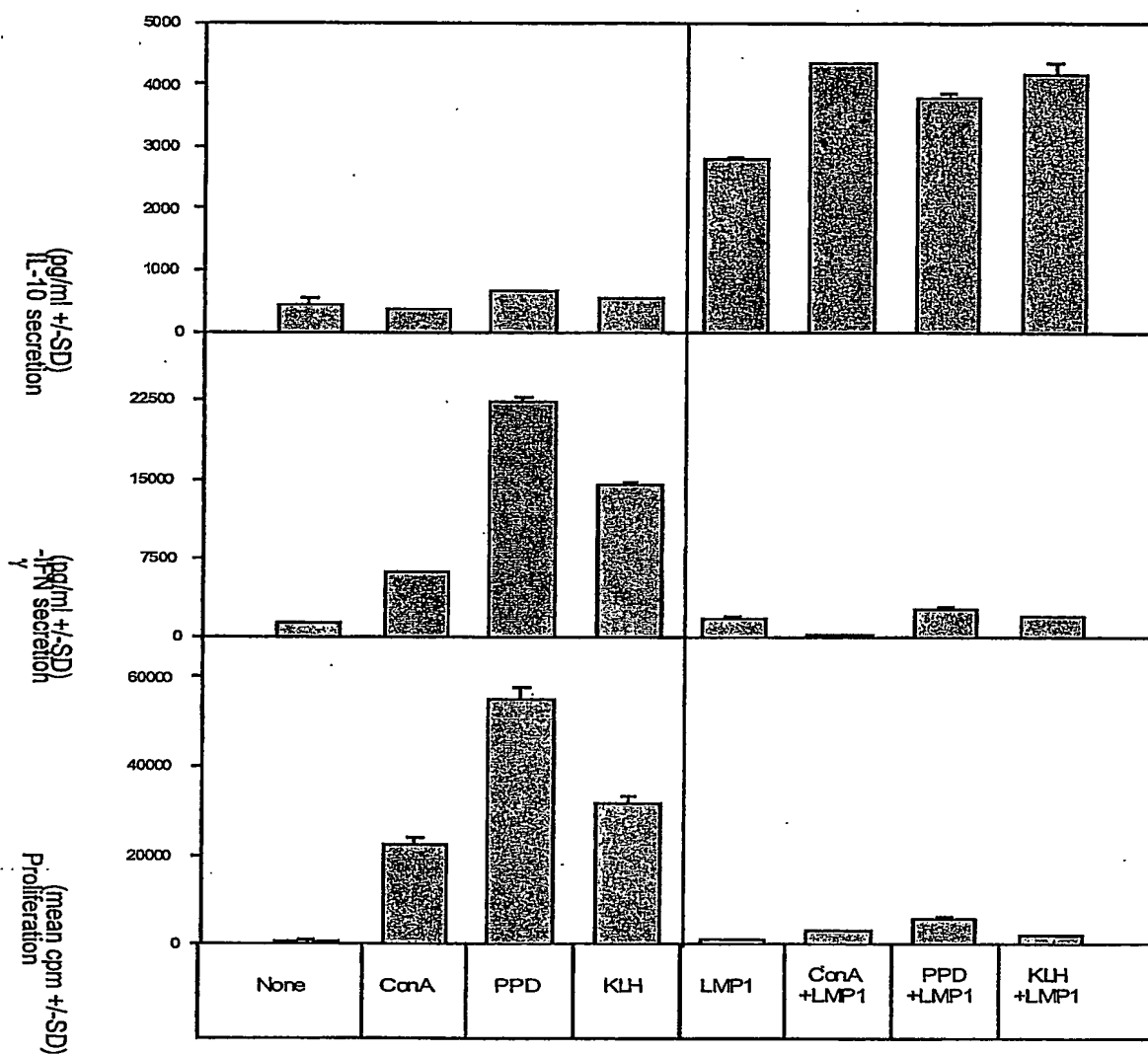


Figure 8a

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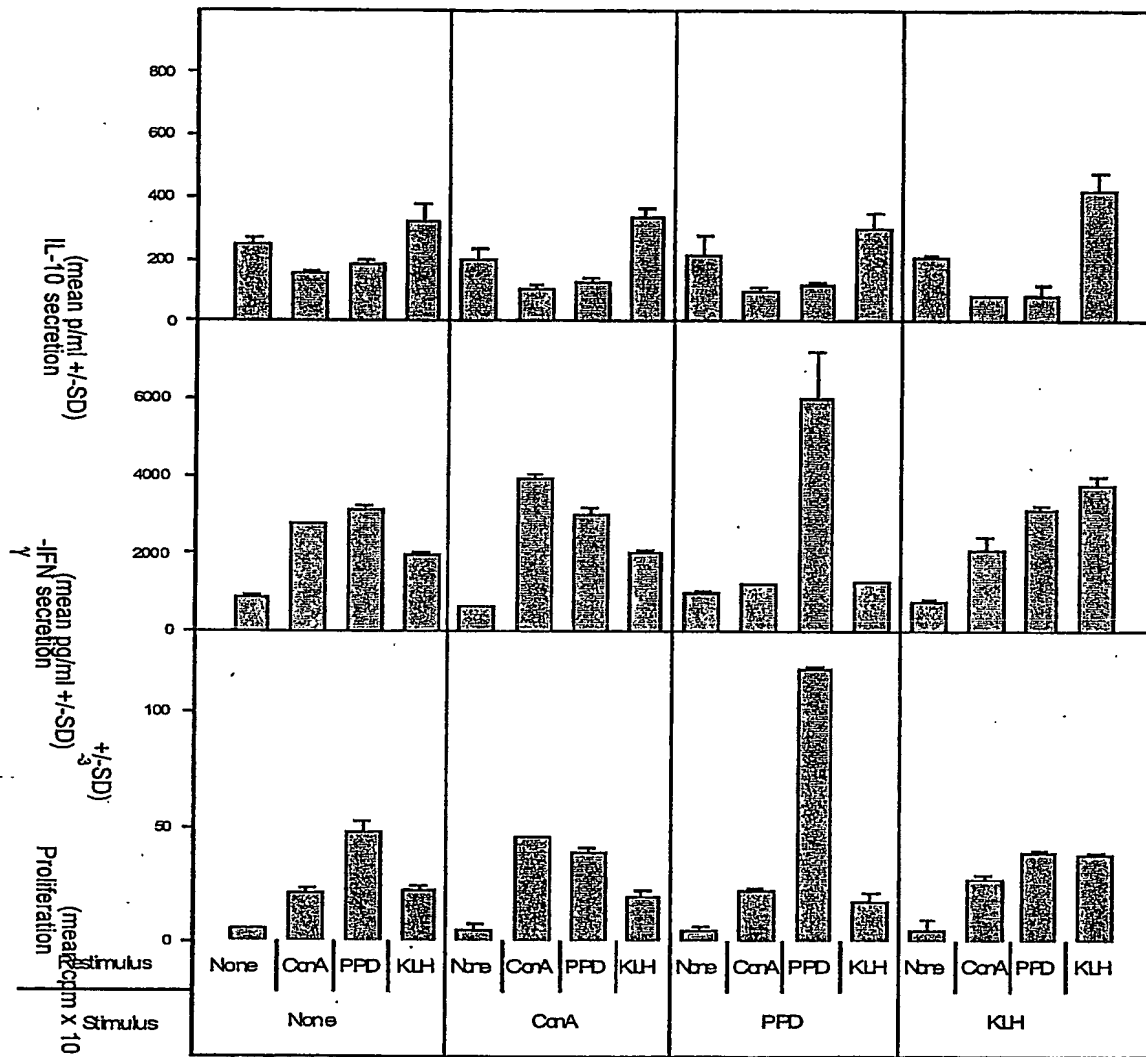


Figure 8b

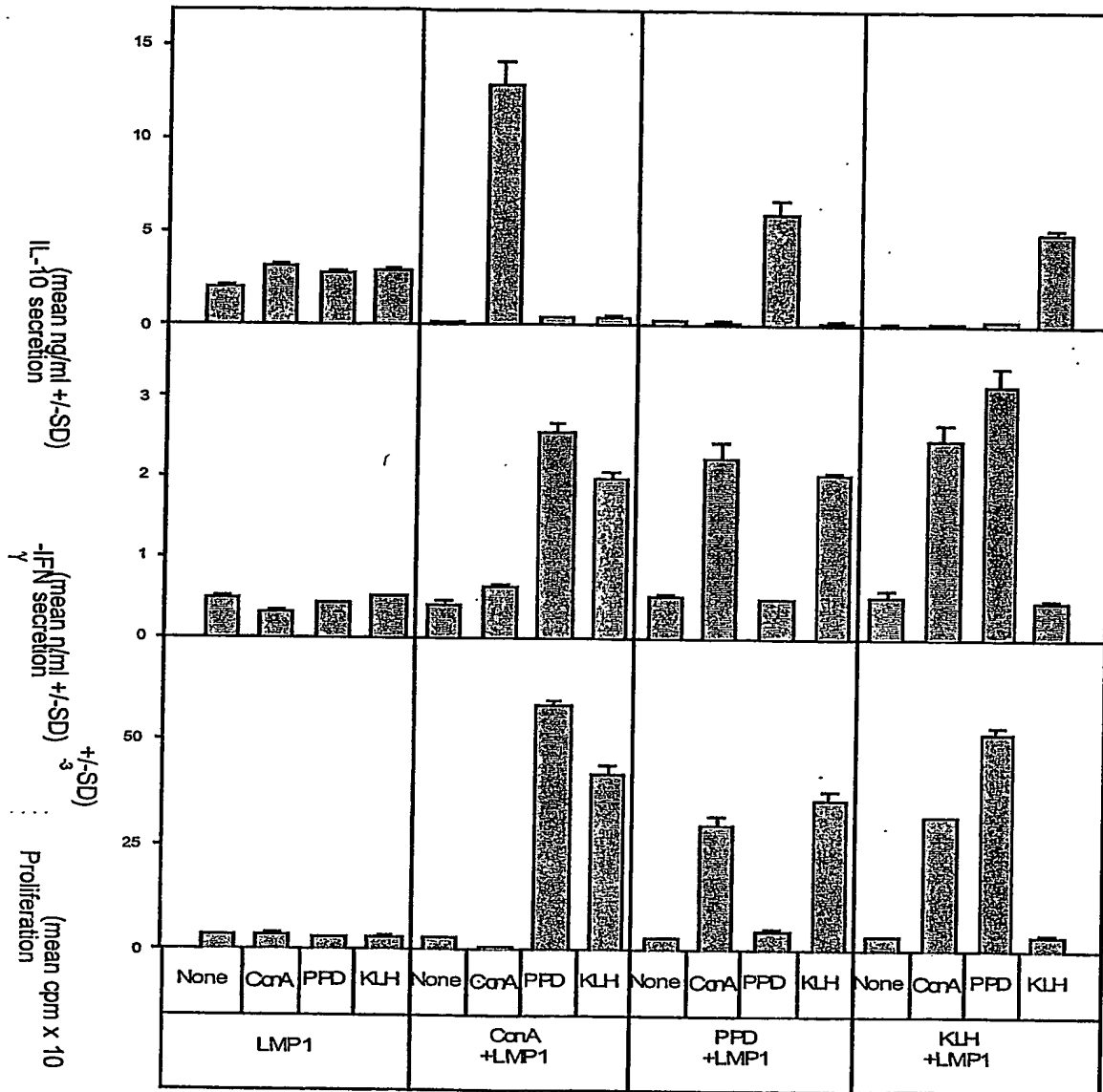


Figure 8c



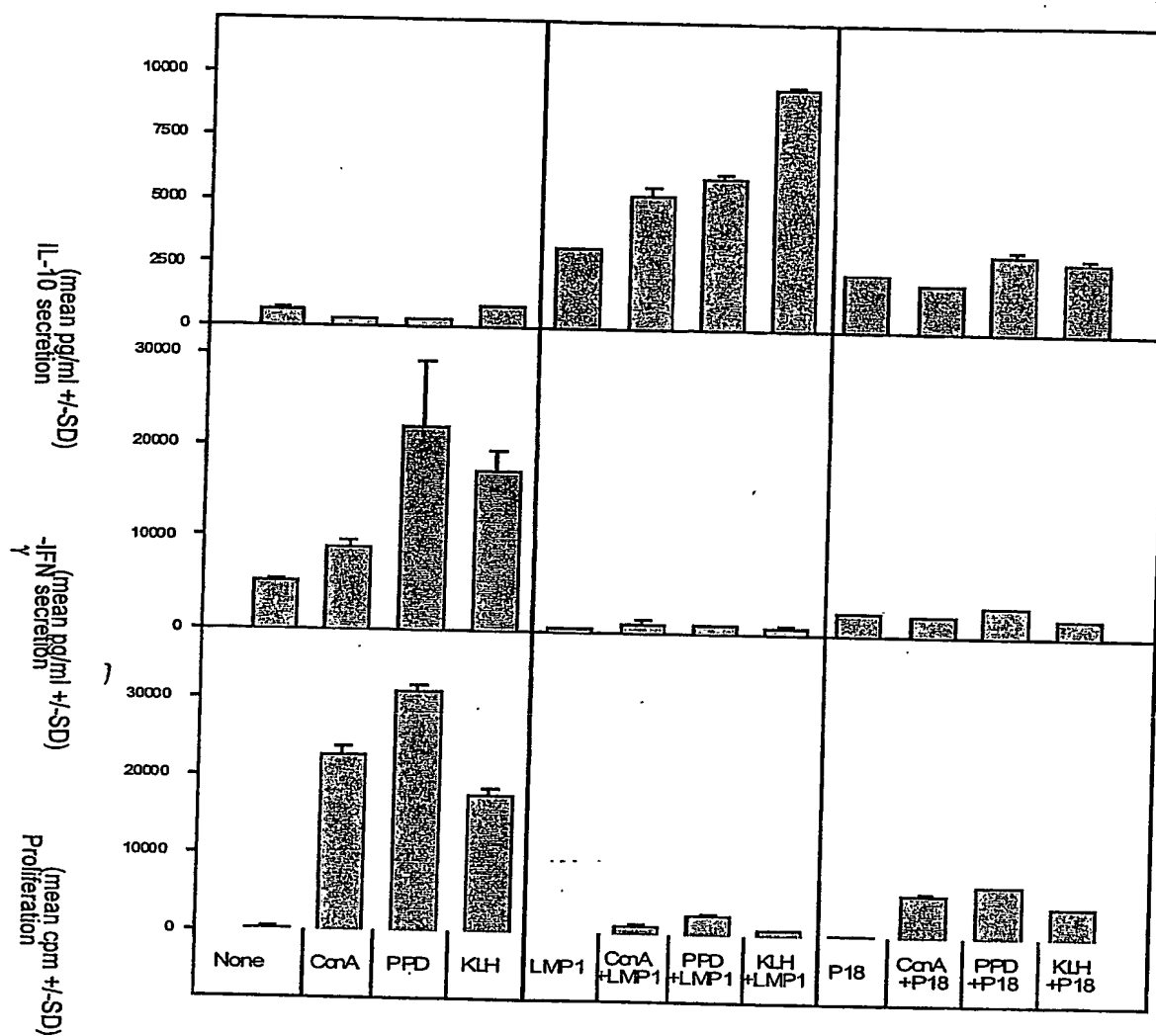


Figure 9a

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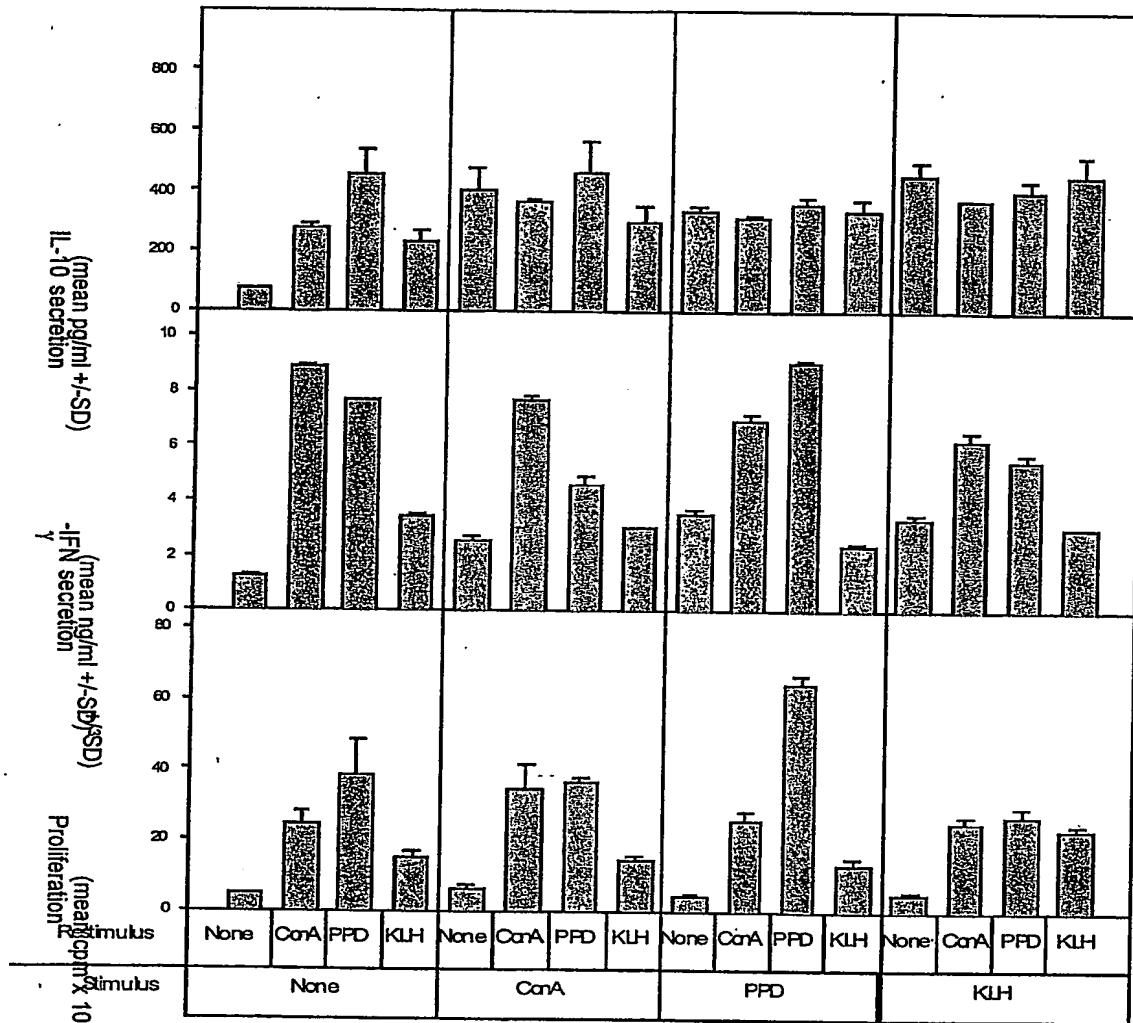


Figure 9b

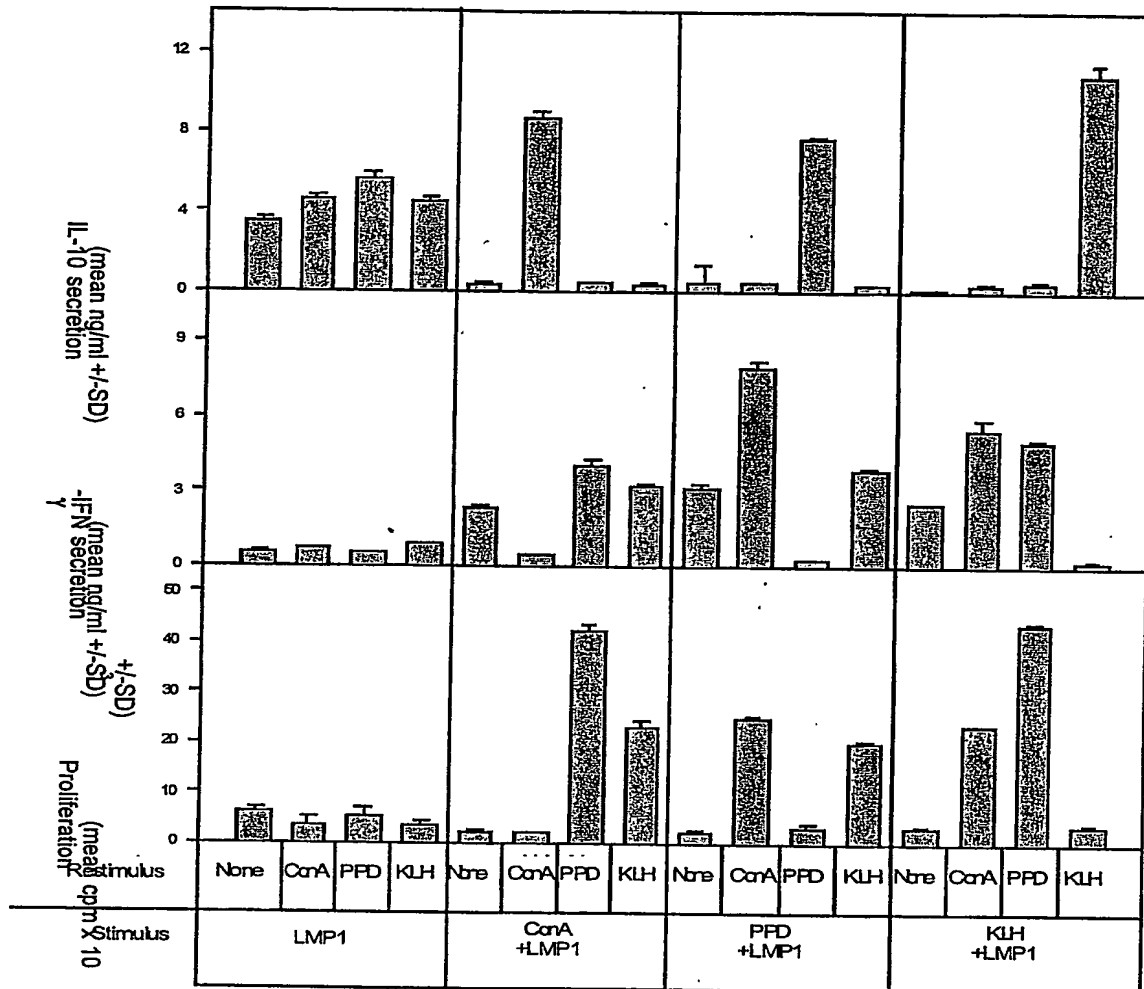


Figure 9c

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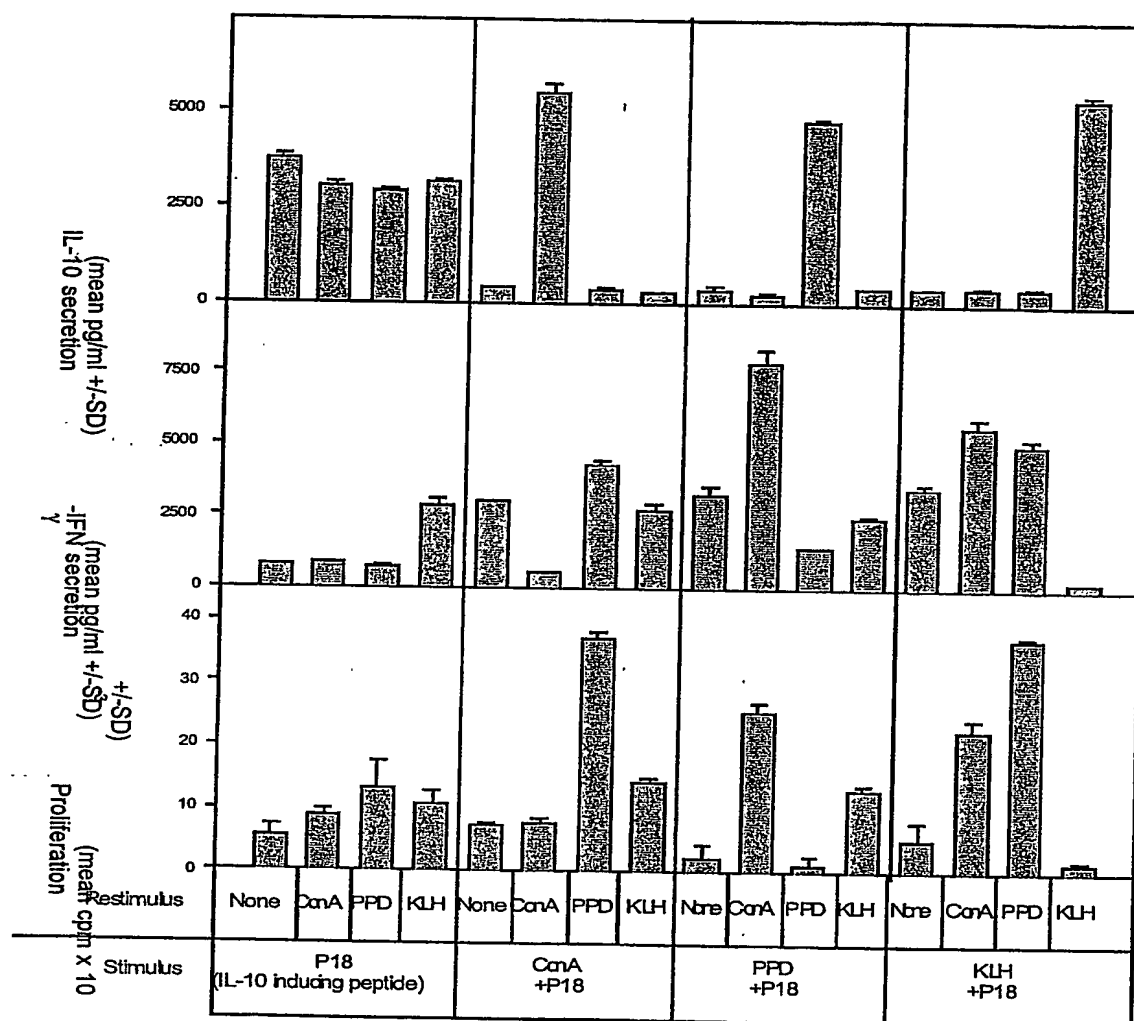


Figure 9d

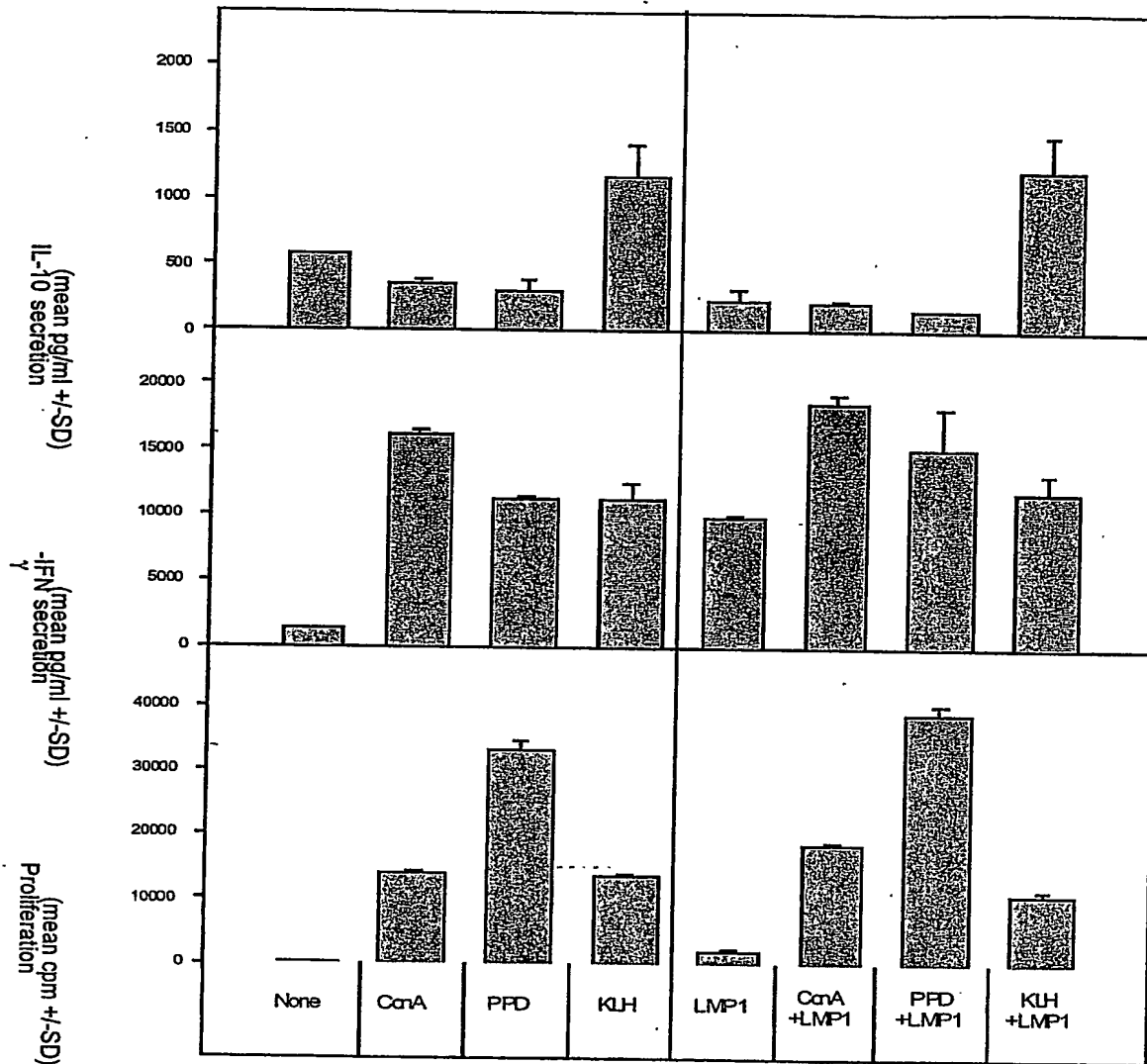


Figure 10a

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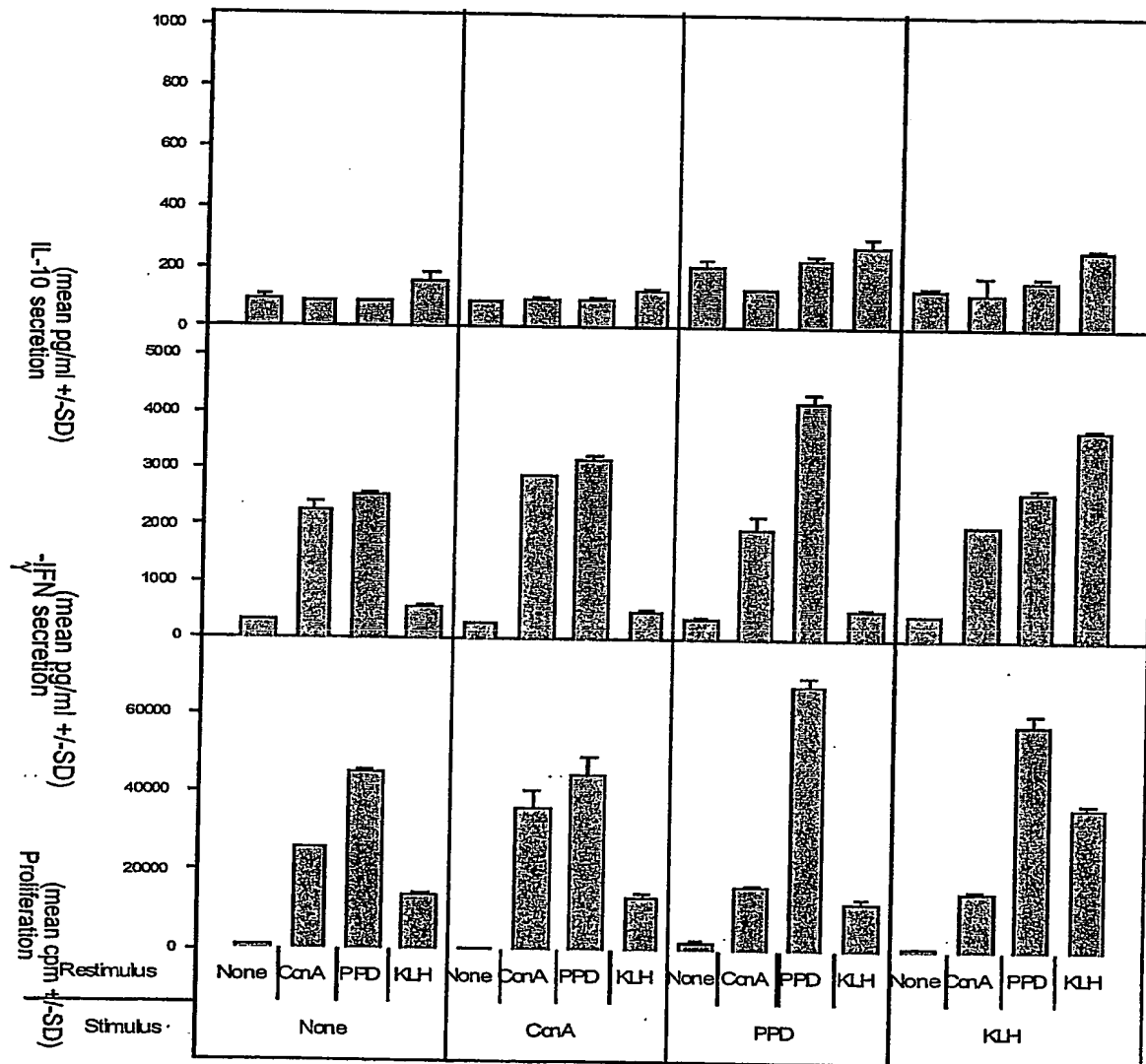


Figure 10b

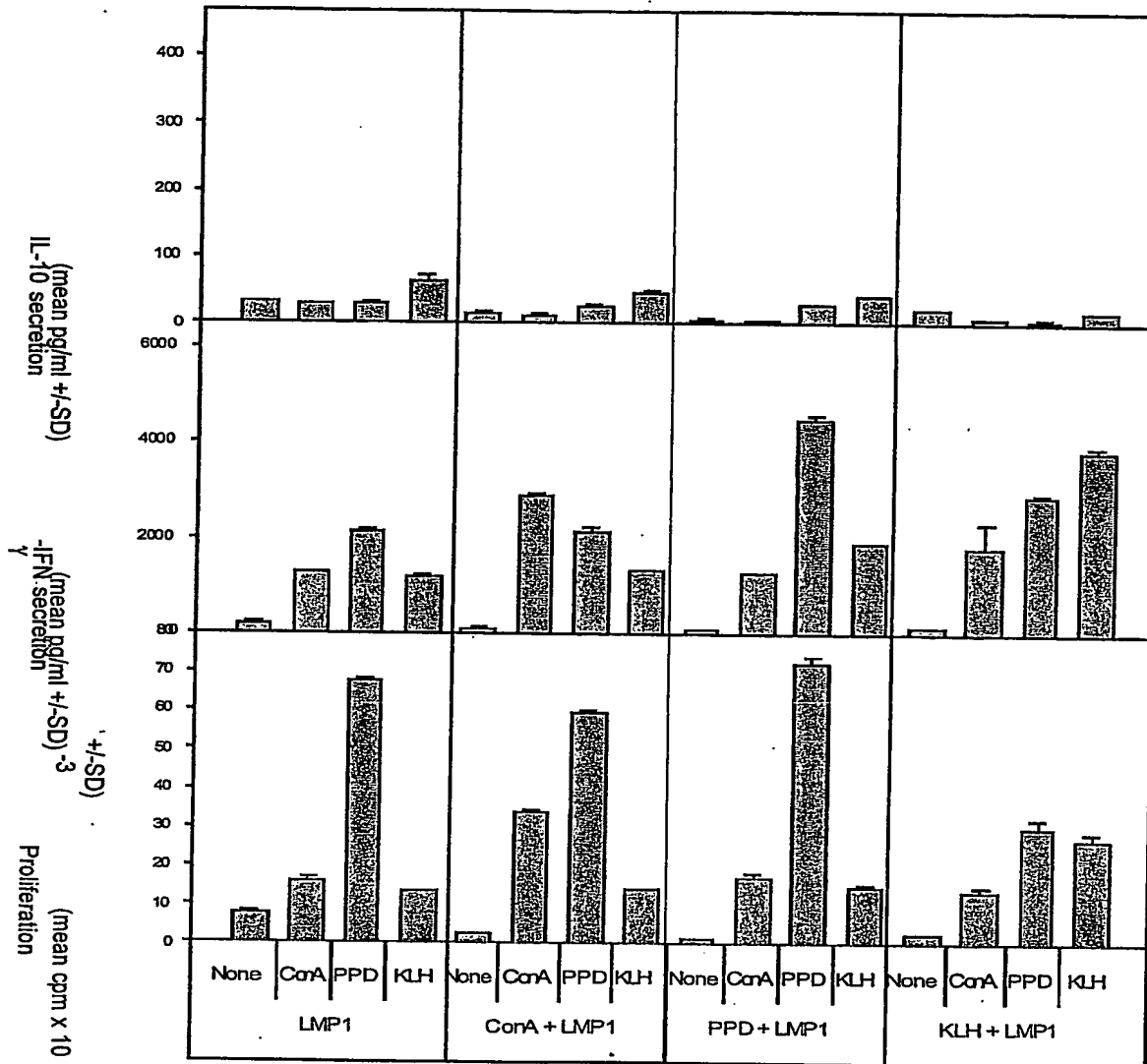


Figure 10c